PALACKÝ UNIVERSITY IN OLOMOUC

Faculty of Science

Department of Analytical Chemistry

Low-cost microfluidic systems for the analysis of biologically active substances

DOCTORAL THESIS

2020 **Mgr. Lenka Hárendarčíková**

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Author: Mgr. Lenka Hárendarčíková

Field of study: Analytical Chemistry

Supervisor: doc. RNDr. Jan Petr, Ph.D.

Olomouc 2020

I hereby declare that I have written the Doctoral thesis myself. All used information and literature sources are indicated in the references.

I agree that this work will be accessible in the library of the Department of Analytical Chemistry, Faculty of Science, Palacký University in Olomouc

In Olomouc .....

……..

Signature

Acknowledgement

I am grateful to my family and companion for their support through my entire doctoral study.

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Lenka Hárendarčíková

Dedication

I would like to dedicate this work to my grandmother for her lifelong support.

ABSTRACT

This doctoral thesis is devoted to development and testing of microfluidic devices from various low-cost materials. The first part of the thesis is dedicated to introduction of microfluidic devices, its history and materials, which are used for their production. Further this part describes production possibilities and detection used in microfluidics. The last part is dedicated to lab-in-a-syringe analysis. The experimental part presents the results of three works. The first part describes the development and testing of laminating film wafer, as an inexpensive alternative to commonly used photolithography, for the production of PDMS devices whose functionality was tested by isotachophoretic separation of two organic dyes - bromophenol blue and amaranth red. The following section describes the development of a low-cost device for multi-parameter chemical analysis of water. This device uses a combination of filter paper with wax printing to create defined channels and reservoirs for individual reactions and a hydrophilic membrane for rapid suction of the sample, the carrier part of the system consists of laminating foils. Selective reagents providing characteristic coloration with the analysed ions are quickly evaluated by the smartphone software application based on HSV (Hue, Saturation, and Value). The application evaluates the amount of analysed ions in the sample (NH_4^+, NO_3^-, NO_2^-, Fe^{2+}, Fe^{3+}, Ca^{2+} and PO_4^{3-}) and pH by comparison with preset calibration values of individual reagents at different analyte concentrations and different sample pH. The last part describes the development of the lab-in-a-syringe test. In this research, we used the experience gained in the manufacture of the previous devices and at the same time enabled detection of the reaction performed directly in the device, which was a major drawback in previously published works. The device consists of a syringe filled with layers of hydrophilic cellulose membrane of various shapes, which serve as a filtration, conjugation and reaction part (detection part). Properties of individual parts of the device were tested on reactions of Ni^{2+} with dimethylglyoxime and Fe^{2+} with 1,10-phenanthroline.

ABSTRAKT

Dizertační práce je zaměřená na vývoj a testování mikrofluidních zařízení z různých nízkonákladových materiálů. První část práce je věnována seznámením se s mikrofluidikou a její historií, materiálům, ze kterých se mikrofluidní zařízení vyrábí. Dále popisuje možnosti výroby těchto zařízení a metody detekce, které se v mikrofluidice používají. Poslední část je věnovaná lab-in-a-syringe („laboratoř ve stříkačce“) analýzám. Následuje experimentální část prezentující výsledky tří prací. První část popisuje vývoj a testování waferu z laminovací fólie, jakožto levnou alternative běžně užívané fotolitgrafie, pro výrobu PDMS zařízení jehož fukčnost byla otestována izotachoforetickou separací dvou organických barviv – bromfenolová modř a amarantová červeň. Následující část popisuje vývoj nízkonákladového zařízení pro multiparametrovou chemickou analýzu vody. Toto zařízení využívá kombinace filtračního papíru s voskovým tiskem pro tvorbu definovaných kanálků a rezervoárů pro jednotlivé reakce a hydrofilní membráni pro rychlé nasávání vzorku, nosnou část systému tvoří laminovací fólie. Selektivní činidla poskytující s analyzovanými ionty charakteristické zbarvení jsou rychle vyhodnocovány pomocí softwarové aplikace chytrého telefonu na základě HSV (Hue - barevný tón, Saturation - sytost barvy a Value - hodnota jasu). Aplikace vyhodnocuje množství analyzovaných iontů ve vzorku (NH_4^+, NO_3^-, NO_2^-, Fe^{2+}, Fe^{3+}, Ca^{2+} a PO_4^{3-}) a pH pomocí porovnání s předem nastavenými kalibračními hodnotami jednotlivých činidel při různých koncentracích analytů a různém pH vzorku. Poslední část popisuje vývoj lab-in-a-syringe testu. V tomto výzkumu jsme využili zkušenosti získané při výrobě předešlých zařízení a zároveň umožnili detekci prováděné reakce přímo v zařízení, což bylo hlavní nevýhodou v dříve publikovaných pracích. Zařízení se skládá z injekční stříkačky naplněné vrstvami hydrofilní celulózové membrány různých tvarů, které slouží jako filtrační, konjugační a reakční část (detekční část). Vlastnosti jednotlivých částí zařízení byly otestovány na reakcích Ni^{2+} s dimethylglyoximem a Fe^{2+} s 1,10-fenantrolinem.

BIBLIOGRAPHICAL IDENTIFICATION

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1. INTRODUCTION

The analytical chemistry is an indispensable part of many industries such as pharmacy, toxicology, food industry, environmental monitoring, and many others. In particular, bioanalytics dealing with the analysis of xenobiotics (substances foreign to the body such as pharmaceuticals, drugs and their metabolites) and biotics (proteins, lipids, nucleic acids, etc.) are often confronted with a limited amount of sample. For this reason, new microfluidic methods have emerged that meet this demand and at the same time are able to deliver robust and reproducible results. First microfluidic chip analysers were developed, these systems are often referred to as the Micro Total Analysis System (μ-TAS). The name μ-TAS expresses an effort to shorten the path of the sample through laboratories and merge as many unit operations as possible into one analyser as big as a credit card. The LOC (lab-on-a-chip) combines all necessary operations such as separation, transport and sample analysis into one chip with channels of several tens or hundreds of micro or nanometers. The microfluidic devices can serve only for pre-treatment of the sample or fully replace conventional analytical instruments providing not only sample analysis but also subsequent detection. We can find their use in practically every area of analytical chemistry.

The dissertation thesis is focused on the development and testing of microfluidic devices from various low-cost materials in connection with colorimetric and smartphone detection. It is based on three original works published in impact factor journals and one patent. One review serves as a basis for the theoretical part. One work is devoted to the development of foil wafer for microfluidic devices made of polymer. The patent describes the development and testing of a portable low-cost device for a multiparameter water analysis. The last work is devoted to the development and testing of the lab-in-a-syringe.

2. THEORETICAL PART

2.1 WHAT ARE MICROFLUIDIC DEVICES?

Microfluidic devices are basically technological systems that manipulate small (10^{-9} to 10^{-18} litres) amounts of fluids into channels with dimensions of tens to hundreds of micrometres.[1] Microfluidic chip is a set of micro-channels etched or molded into a material (glass, silicon, polymer etc.), connected together in order to achieve the desired features for fully automated analytical system, such a devices are also known as Micro Total Analysis System (μ-TAS). Comparison between size of a micro- or a nanofluidic device with other common entities is shown on Fig. 1

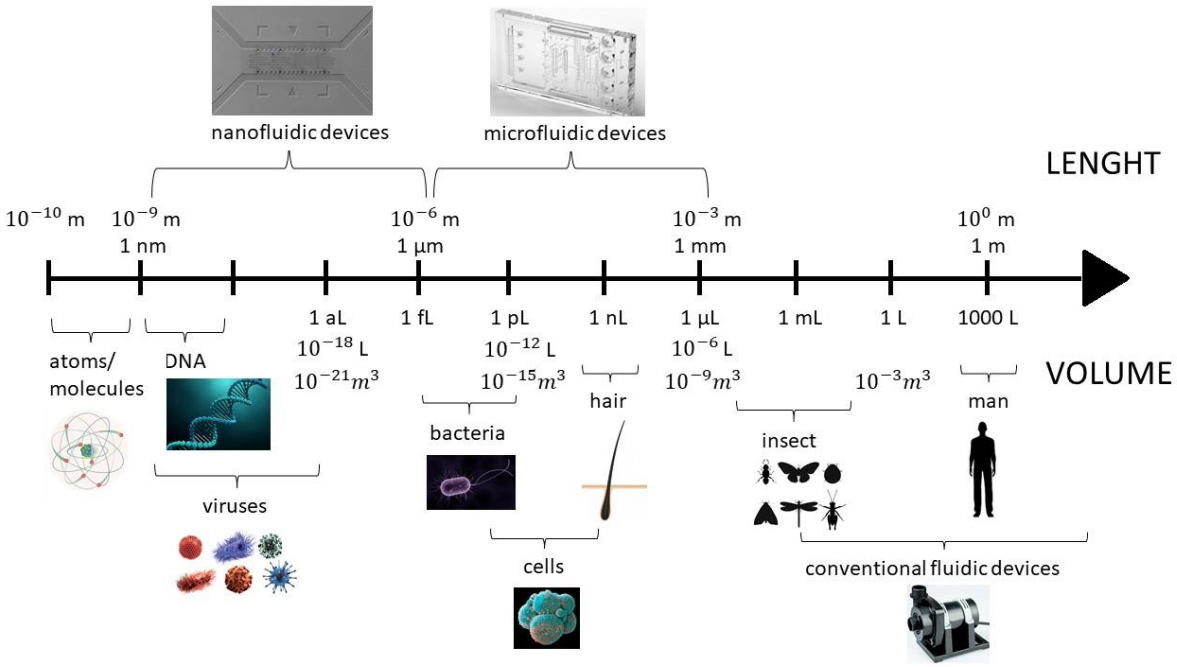

Figure 1. Comparison between size of a micro- and a nanofluidic devices with other common entities

This technology started with trend of miniaturization of analytical methods within approx. last 30 years. First of all, the analysers were downgraded to apparatus, which were able to be placed on working table. Subsequently the hand (portable) analysers have appeared. In recent years, we have been going over to analysis on chip, so called Lab-on-a-chip (LOC).

Micro and nanochips are final stadium of this process nowadays. Miniaturization has numerous advantages: e.g. reduction of acquisition and operating costs (energy consumption, reagent volume, etc.) of individual devices, easy handling and controlling, spatial ease in the lab and beyond. All these aspects lead to cheaper price of the lab analysis. We still have to consider all the processes so that it will not lead to a reduction of the quality of the obtained results. Therefore, all microfluidic devices are developed to be able to compete with instrumental methods by their detection limits and other analytical characteristic. [2].

2.2 WHAT WERE THE FOUNDATION STONES OF MICROFLUIDICS?

In 1949, Müller and Clegg patterned a filter paper with a paraffin barrier and observed speeding up of the diffusion process of the sample caused by the confined channel and reduced sample consumption, therefore this research was regarded as one of the basis of paper microfluidics. [3] In 1956, the first paper device for the semi-quantitative detection of glucose in urine was presented by J. P. Comer.[4] On the other hand the first microfluidic application in industrial environment occurred in 1980s and were triggered by the generalization of ink jet printing and micro electromechanical systems (MEMS) used for example in pressure sensors. In 1979, Hewlett Packard developed the Thermal Inkjet Technology and MEMS technology used to manufacture the nozzles. [5,6] The microfluidic appeared during last hundred years in various disciplines, but based on professor Whitesides theory, it originated in following four fields: molecular analysis, biodefence, molecular biology and microelectronics. First were analysis, as mentioned above, there was trend of miniaturization in all analytical methods like GC, HPLC and CE, which in capillary format revolutionized chemical analysis. The second motivation came from the field of molecular biology. In this discipline the need of analytical methods with much greater throughput and higher sensitivity and resolution for DNA sequencing and other genomics emerged in 1980s. Best solutions to overcome these problems were offered by microfluidics. Third motivation was danger of chemical and biological weapons after the cold war. In 1990s, Defense Advanced Research Projects Agency (DARPA) of the US Department of Defence supported many programmes aimed at developing portable microfluidic systems for detection of chemical and biological threats. The last contribution was from microelectronic, where photolithography and associated technologies were successful

in silicon microelectronics and MEMS. The original motivation was to directly apply them into microfluidics. In 1990 also Manz et al. presented Miniaturized Total Analysis System (μTAS), which periodically transforms chemical information into electronic information. Sampling, sample transport, chemical reactions, chromatographic separation and detection are automatically carried out in such a device. The detector or sensor in a TAS does not need high sensitivity, because sample pre-treatment serves as elimination of most of the interfering chemical compounds and impurities. Calibration can be incorporated in the system as well.[7] Some of the earliest works used silicon and glass in fluidic microsystems, but later polymers became the most widely used materials for microfluidics fabrication. [8–11]

2.3 WHICH MATERIALS ARE USED IN MICROFLUIDICS?

Some of the materials were already mentioned in previous chapter, but here I would like to mention more widely their use in recent and past applications mostly in connection with smartphone detection, which will be later used in experimental part of this thesis. Much of this chapter was drawn from my review and the literature sources used in it, that's why this part discusses the materials together with smartphone detection, which will be later used in experimental part – chapter 4.2, another types of detection are discussed separately in chapter 2.5.[12]

Paper

The origin of paper-based microfluidic devices has been attributed to the Whitesides Group (Harvard University) where the "lab-on-a-paper" was developed. Paper devices are commonly used in many areas. Water composition testing is one of the most needed analyses, especially in Third World countries where water quality is poor. The parameters of this analysis, such as metals, organic pollutants, pesticides, bacteria and viruses, and other toxic substances, are monitored mostly using instrumental methods (e.g., GC, spectrometry, potentiometry, conductimetry, AAS, ICP-MS, immunoassays, and the like) but also microfluidics. [13–22]

In 2014, Lopez-Ruiz et al. [20] used smartphone-based colorimetric detection for simultaneous pH and Nitrite determination on a paper-based microfluidic device (Fig. 2). Their device used seven sensing areas with immobilized reagents, which produced selective color changes after the sample from the middle part reached the sensing area. After 15 min, a picture of the device was taken by Samsung Galaxy SII with dedicated software (built-in camera parameters were fixed in the developed application to avoid automatic configurations). The picture was taken with flash in the torch mode, which served as a light source under controlled conditions. Although the microfluidic device was placed in the same position during the acquisition procedure, some differences between angle scales could appear. To resolve this situation the real distance of the triangle and the square was included in software settings, so the application knows the distance and the angle of shooting. In short, it can be said that the above analyses were performed in three steps: detection of the marks (triangle and square), detection of the sensing areas, and color analysis by the customized algorithm. Afterwards the application showed the results of pH and nitrite concentration in mg/L. The accuracy of pH determination was 0.09 and the resolution 0.04 units of pH. LOD for nitrite determination was 0.52 mg/L.

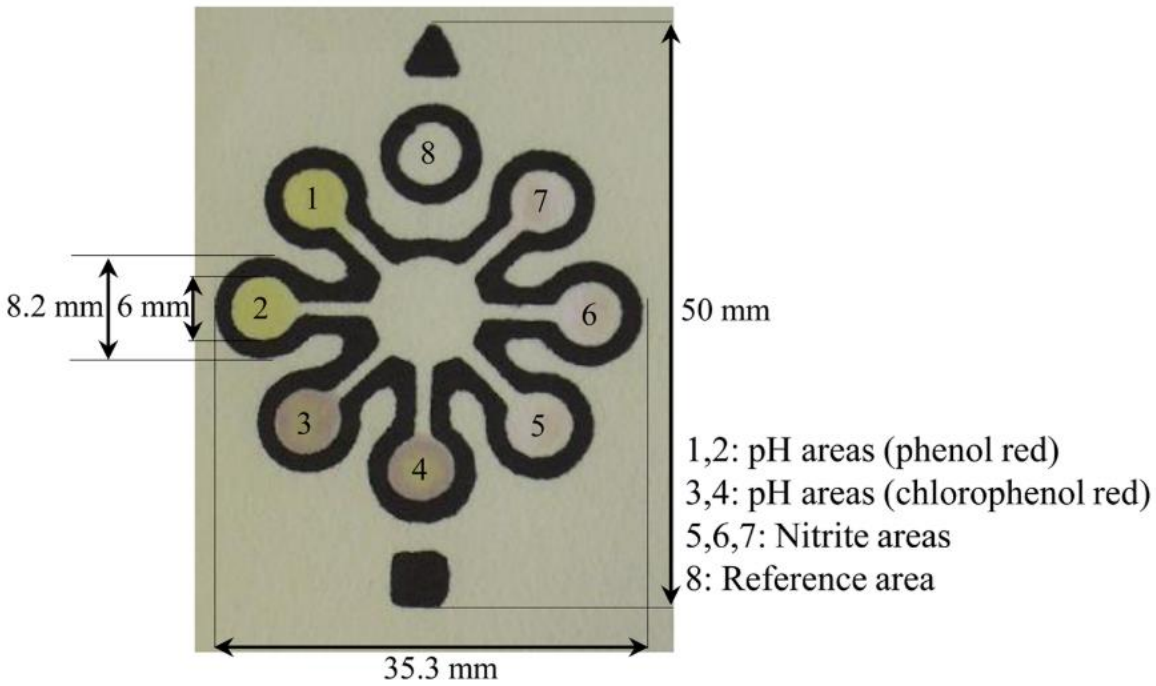

Figure 2. Microfluidic device for pH and nitrite determination [20]

Almost the same device as the one described by the Parks group used for the evaluation of the taste of ten different red wines, with the use of a set of chemical dyes. [23] Since colorimetric detection was discussed in the previous chapter, it is appropriate to explain that colorimetric detection in microfluidic can be conducted in reflectance or transmittance mode. The reflectance mode is used mostly in paper-fluidics and can be accomplished by a facile manner using a standard smartphone and freeware such as ImageJ or using own designed software, for example, one coded in MATLAB.

Wang et al. [24] developed a 3D paper-based device for colorimetric determination of Ni(II), Cu(II), Cd(II), and Cr(VI) in water sample with detection limits around 0.3 ppm. The device was fabricated by stacking layers of wax-patterned paper and double-sided adhesive tape, which made it capable of wicking fluids and distributing samples from single inlet into the affrays of detection zones without external pumps. The tape layers were patterned by holes, which were filled with cellulose powder and reagents. This connected the channels in different layers of the paper. This device in combination with a smartphone camera as a detector represents the first step to multiplex heavy metal detection in environmental monitoring.

Bacterial measurement is equally important in water analysis. A single-cell-level detection paper microfluidic was successfully demonstrated by Park et al. [25]. Their device consisted of three channels pre-loaded with BSA-conjugated and anti-*Escherichia coli*-conjugated beads with varying amounts (negative control, low- and high-range *E. coli* detection). A sample passage was driven by capillary flow and two pictures were taken for the final evaluation by a smartphone camera, the first one before wastewater sample loading and the second one thirty seconds after the sample loading. *E. coli* antigens caused immunoagglutination of antibody-conjugated beads, which was quantified by evaluating Mie scattering from digital images taken at an optimized angle and distance of the cell phone. Both images were analysed using a program coded in MATLAB, where the code algorithm converted the color image to a green image, recognized channel region, and calculated average intensity. Each sample signal was divided by the background signal to cancel out the difference between the paper chips. The same evaluation principle was used in a multichannel device for *Salmonella* and *E. coli* measurements in one device. This system had an advantage over the previous system because it did not require any holder for the cell phone or a sensor. Park et al. repeated the above mentioned experiments for *E. coli* determination also with UV LED

as a light source. The UV LED (385 nm) irradiated the paper chip at the incident angle of 30° to the chip surface and the smartphone took an image at 25° from the chip surface. Pictures both before and after the sample loading (E. coli in DI water, and spiked one with 10% of human whole blood) were split into red, green, and blue channels. The results showed that the intensities of the green pixels were most sensitive. The intensities from the positive channel were compared with the negative channel and evaluated (the device consisted of channels pre-loaded with BSA-conjugated and anti-E. coli-conjugated beads with varying amounts used for negative control, low- and high-range E.coli detection for positive control). In this research, Park's group demonstrated the significant enhancement of Mie scatter signals of particle immunoagglutination assays on paper chips using UV LED. Normalized signal intensities increased up to 50% for E. coli assay, a tenfold improvement from the same assay with ambient light even with 10% of human whole blood in the sample. [26,27] The classical microPADs (microfluidic paper-based analytical devices) production is based on a wax ink printing to form a hydrophobic channel in paper as mentioned above. But Strong et al. came in 2019 with novel approach to production of higher resolution and high-fidelity microPADs. They immersed standard microPADs with dimensions 4.5x4.5 cm into sodium periodate ($NaIO_4$) solution with concentration of 0.3, 0.4, 0.5 and 1.0M at the room temperature in Petri dish. The Petri dishes were shielded from ambient light during the reaction, which takes from 6 to 96 hours. Thereafter, the devices were rinsed with DI water, dried and then measured with a ruler. This treatment miniaturized microPADs by up to 80% in surface area, depending on the concentration of $NaIO_4$ and length of reaction time, for example after immersing microPADs in 0.5M $NaIO_4$ for 48 hours were devices miniaturized by 78% in surface area and fabrication of functional channels with widths as small as 301 μm and hydrophobic barriers with widths as small as 387 μm were achieved.[28]

Test-stripe

The test-strip-based devices using test strips made of different materials in combination with colorimetric detection have been used most commonly. Test strips are often placed in a plastic case or a cassette to avoid penetration of daylight which can cause measurement errors. The case also contains a flash diffuser. For example, Oncescu et al. [29]

used this approach for cholesterol testing in blood samples. Within sixty seconds, their device could accurately quantify total cholesterol levels in blood by imaging the standard test strip and looking at the saturation of the acquired image. Addition to this, they also demonstrated the abilities of their system to identify erroneous readings, which current commercial devices cannot do. With the use of the smartCARD system, they were also able to measure other commercially available colorimetric test strips for LDL, HDL cholesterol, and triglycides.

Same group also developed a health accessory for colorimetric detection in sweat and saliva. They measured the pH in saliva, which can be correlated to sodium concentration and the sweat rate in order to indicate to users the proper time of hydration during physical exercise, to avoid the risk of possible muscle cramps. Their device was isolated from ambient light with a hardware accessory and diffused the light from the smartphone camera flash for reproducible and uniform illumination. The system consists of a smartphone case with a slot for the test strip, application, and the test strip. For the measurement, a user has to lick the test strip or wipe sweat with test strip and place it into the case. The test strips incorporate an indicator strip, a reference strip (made of white plastic in order to detect changes in white balance on the iPhone camera) and a flash diffuser (consisting of PDMS membrane) inserted in 3D printed support (made of opaque black material). The indicator strip consists of a pHydrion Spectral 5.5 to 9.0 plastic pH indicator strips for sweat testing and a 1.0 to 14.0 strip for saliva testing. The image from the smartphone camera is defined by RGB values, but the individual red, green, and blue channels are not correlated well with the pH over the range of a universal indicator strip. It can be, however, converted to hue values which match the color spectrum of the test strip more closely. A hue was chosen also because it is more precise than RGB values for quantitative analysis and less sensitive to variation in illumination, as Cantrell´s group discovered. [30,31] More recent application of smartphones and paper based microfluidic devices are mentioned in Table 1. Common test strip for detection of hCG (human chorionic gonadotropin) – pregnancy test is shown at Fig. 3. The hCG is glycoprotein hormone and it is produced by the placental syncytiotrophoblast during pregnancy.

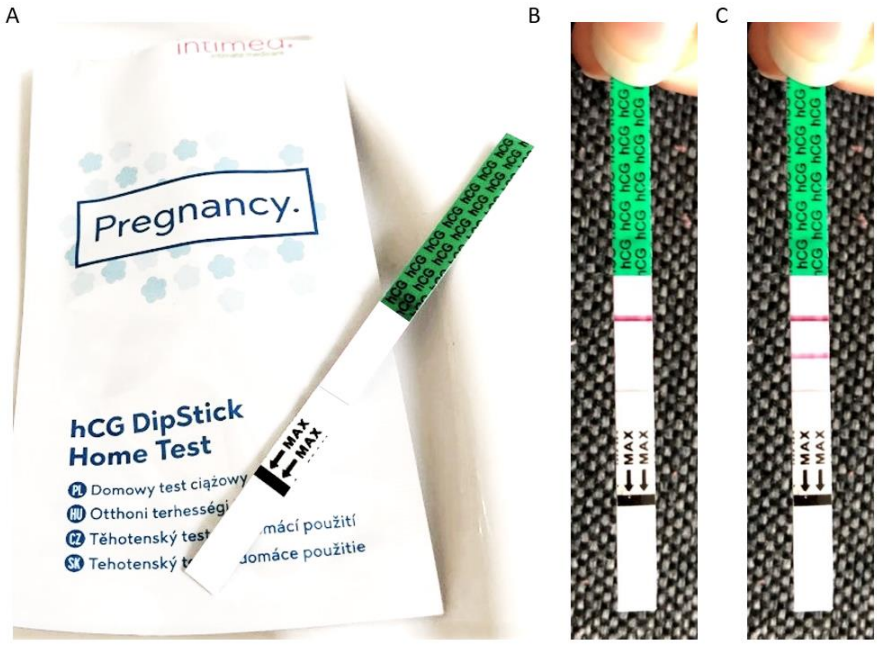

Figure 3. Pregnancy test: (A) unused test, (B) negative test, (C) positive test

Table 1: Recent applications of smartphones and paper-based microfluidic devices

Material of the chip	Application	Ref
Paper	Antibody based determination of E. coli and Neisseria gonorrhoeae, smartphone used for colorimetric detection, analysed in the ImageJ software	[32]
Paper	Determination of glucose and lactate, acryl case for standardization of light intensity	[33]
Paper	Blood-type analysis, smartphone used for readout the results	[34]
Paper	Detection of ion concentration by fluorescence image recorded by a smartphone	[35]
Paper	Colorimetric detection of acid-base titration on paper-based microfluidic support	[36]
Paper	Smartphone detection optimization including correction to illumination for water quality detection using test strips	[37]
Paper, integrated device	Detection of influenza A based on ELISA with smartphone for image processing, custom software written	[38]
Paper, integrated with optoelectronics device	Micronutrient quantification (Ferritin, RBP, CRP, and PC assay) based on immunoreactions, integration to electronic device with external driver based on smartphone (smartphone is used only as a controller)	[39]

Table 1 Continued

Paper, PDMS, chromatographic paper	A folded polydimethylsiloxane (PDMS)-coated paper mask with a specific pattern to form a sandwich structure with inserted chromatographic paper creates μPAD for detection of melamine	[40]
Paper	Trapezoidal distance-based microfluidic paper device for argentometric determination of chloride	[41]
Paper	3D paper-based microfluidic electrochemical biosensor for glucose determination	[42]
Paper	Laser printed μPADs for point-of-care applications (NO_2^-, E. coli)	[43]

Polymer

Polymers became the most widely used materials for microfluidics fabrication due to their low cost, optical transparency, mechanical and chemical properties, and simple fabrication procedures [9]. However, in combination with smartphone detection, they offer many challenges. Jiang et al. [44] reported a low-cost microfluidic EIS (electrochemical impedance spectroscopy) bacteria pre-concentrator and sensor based on a smartphone through wireless connection, which enables to detect the density as low as ten *E. coli* cells per 1mL water. The sample solution was injected from the bottom inlet, large particles were blocked by a silicon filter, and the bacteria of interest passed through the micro-hole silicon filter and were blocked by a nonporous filter above the interdigitated sensing electrodes. The wireless system included an Android cell phone, a Bluetooth shield, a microcontroller, a chip for impedance converter network analyser, and their packed sensor. Figure 4 shows all the system parts mentioned above. The basic diagram of the wireless inductance, capacitance, and resistance sensing platform which communicates with the mobile phone and drives the packaged bacteria sensor to quantify the bacteria is illustrated in Fig. 4d.

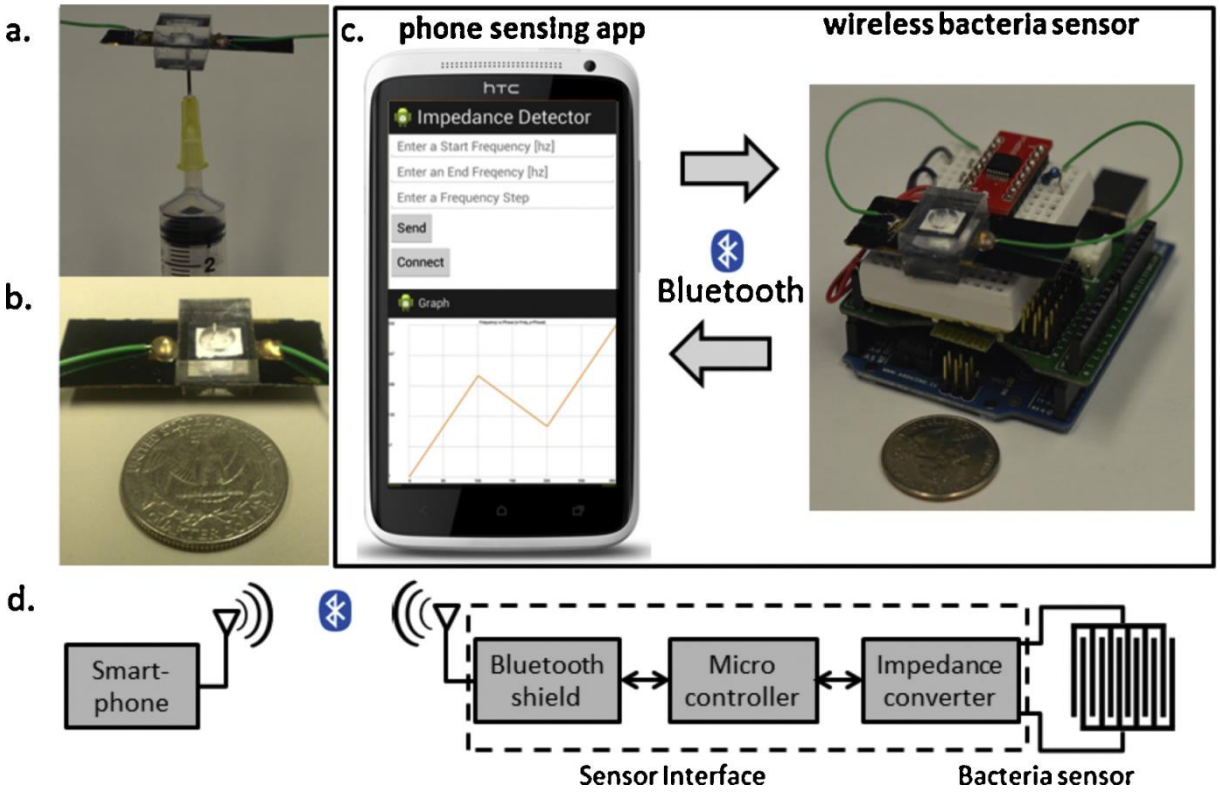

Figure 4. Wireless mobile phone bacteria sensing system. (a) Picture showing syringe injection of testing liquid into the sensor package; (b) close view of the EIS bacteria sensor package; (c) picture showing communication scheme between smartphone sensing app and wireless bacteria sensor; (d) diagram of wireless sensing system [44]

The system consisting of a smartphone accessory, an app, and a test strip for colorimetric detection of 25-hydroxyvitamin D (25(OH)D) was used for gold nanoparticle (AuNP)-based immunoassay determination by Lee et al. [45]. The test strip was coated with 25(OH)D and the sample (modified blood serum) was applied onto the detection area, with the result of only the antibody conjugates not bound to the 25(OH)D captured on the surface. The colorimetric signal from AuNP–antibody conjugates was then amplified using silver, which was reduced on the surface of AUNP to increase their size and the sensitivity of the system (see Fig. 5). The whole test strip was placed in the smartphone accessory (vitaAID accessory) to minimize the effect of variability in external lighting and embedded in a PDMS diffuser to illuminate the back of the test strip. Once the competitive binding of AuNP–anti-25(OH)D was performed, the colorimetric change was captured using the smartphone's camera after the insertion of the test strip in the vitaAID accessory. The concentration of 25(OH)D was estimated by comparing the detection and reference areas. The approach using the AuNP-

labelled microfluidic immunoassay with a camera of a cell phone as the detector was published by Lu et al. [46]. The heterogeneous immunoassay between anti-human IgG and human IgG was performed.

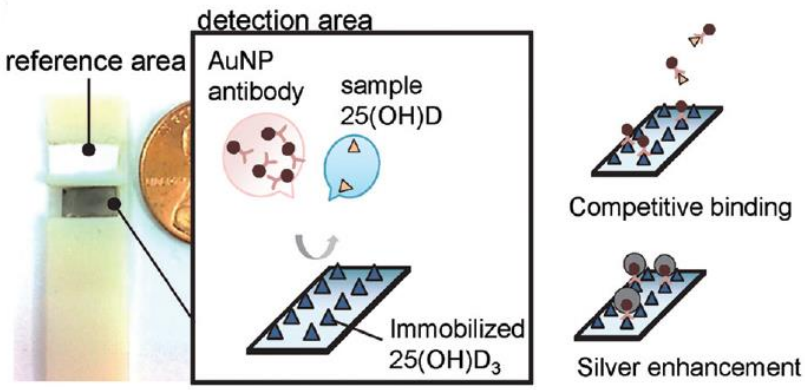

Figure 5. Test strip and schematic of the gold nanoparticle–based immunoassays reaction on the detection area [45]

Kim et al. [47] developed a smartphone-based optical platform for colorimetric analysis of blood hematocrit. It consisted of a white PMMA box including a PDMS light diffuser. A microfluidic device was placed in the bottom part and a smartphone was placed on the top of the white box. The principle of light diffuser is shown in Fig. 6a and b. A schematic view of the whole developed platform and the light diffusion effect is shown in Fig. 6c. The images of the blood which was contained in the microchannels was taken by the smartphone integrated camera and analysed by the image-processing mobile application by calculating the relative GSV of each image pixel. GSV means gray-scale-valuation, which convert color images to grayscale. The main reason why grayscale representations are often used is that grayscale simplifies the algorithm and reduces computational requirements. The given color may be of limited benefit in many applications and introducing unnecessary information could increase the amount of training data required to achieve good results. In this case, the relative GSV of blood hematocrit was determined by subtracting the averaged GSVs of the microchannel from the reference GSVs of the substrate according to the Eq. (1):

$$\boldsymbol{Average\ GSV} = \frac{\boldsymbol{\Sigma r_{av}+g_{av}+b_{av}}}{\boldsymbol{3}}, \qquad (1)$$

where, r_{av}, g_{av}, and b_{av} are the average of red, green, and blue for the total pixels of the selected region of the image.

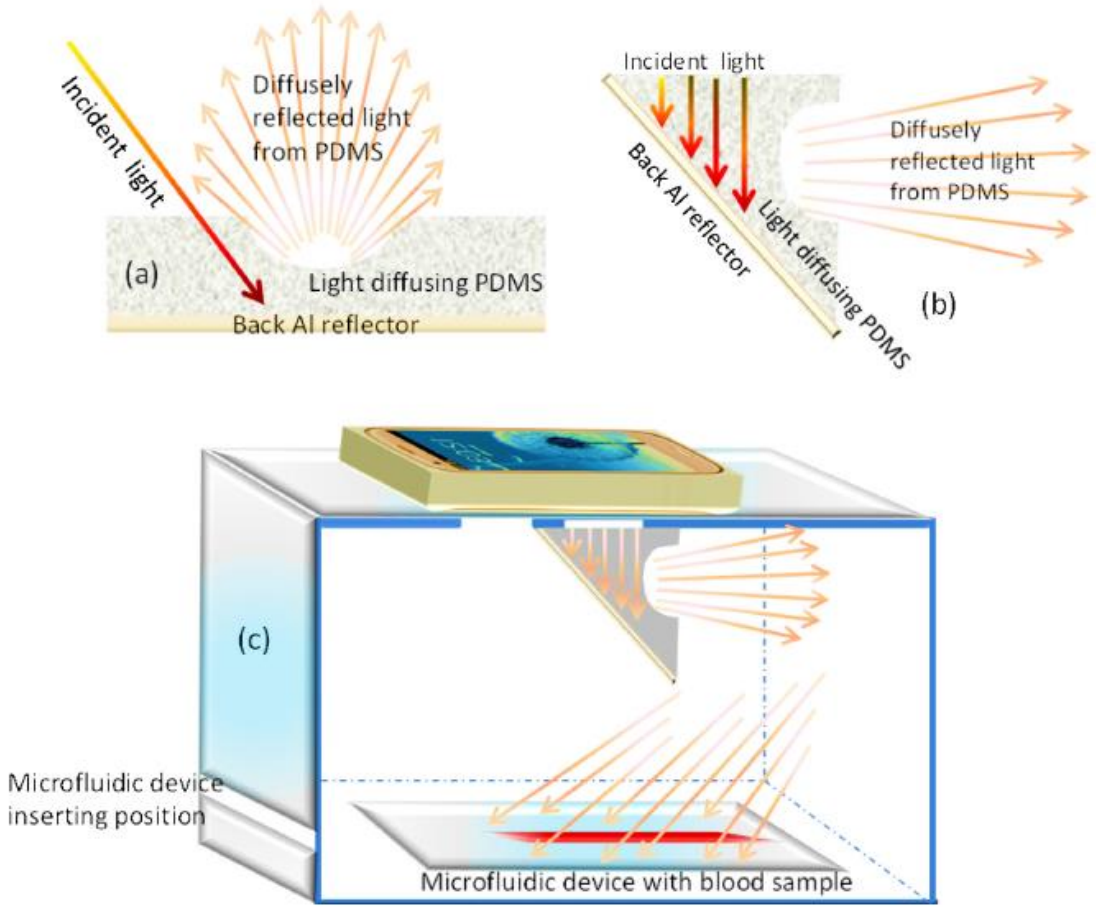

Figure 6. (a) and (b) light diffusion effect from U-shaped PDMS placed on a reflecting aluminium back contact; (c) schematic view of the whole developed platform and the light diffusion effect [47]

A similar approach to the one used by Park et al. [27] in the paper device was used in a PDMS device for the detection of infectious pathogens directly from diluted (10%) human whole blood. [48]

Lillehoj et al. [49] introduced a different PDMS device for determination of HRP-2 using electrochemical detection (see Fig. 7). For sample loading, processing, and pumping, the capillary flow was employed in the used device. HRP-2 captured antibodies were immobilized on the electrode surface within a robust polypyrrole matrix and the detection was achieved by combining a highly specific protein-binding scheme with high sensitivity

enzymatic amplification. The reporter solution contained HRP-2 detection antibodies conjugated to horseradish peroxidase in Dublecco's PBS. When HRP-2 was not present in the sample, the detection antibodies were unable to bind to the surface and were washed away when the TMB/H_2O_2 (3,3',5,5'-tetramethylbenzidine/hydrogen peroxide) substrate was loaded into a chip. In the absence of HRP-2, a negligible electrochemical current was generated when a voltage potential was applied. In the presence of HRP-2 antigen, the detection antibodies bound to the sensor surface and the application of the TMB/H_2O_2 substrate with a voltage potential resulted in a substantial electrochemical current directly proportional to the concentration ofHRP-2 protein in the sample. Figure 8 shows the illustration of the electrochemical detection. Complete measurement was achieved within 15 min and lower detection limit in human serum was 16 ng/mL.

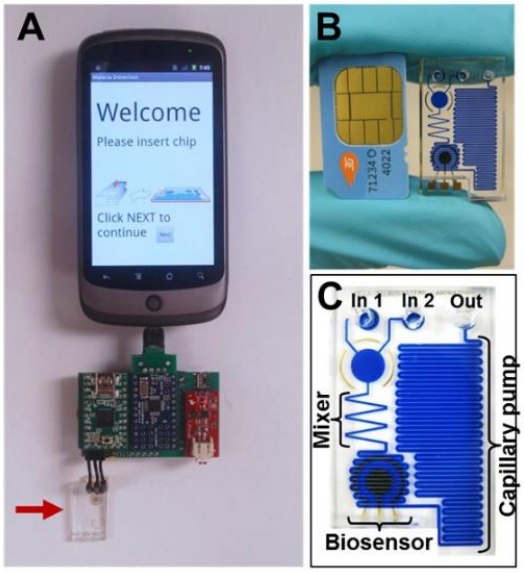

Figure 7. (A) Photograph of the prototype, (B) Size comparison with a SIM card, (C) An enlarged chip image with labelled components - channels were filled with dye for improved visualization of the fluidic network [49]

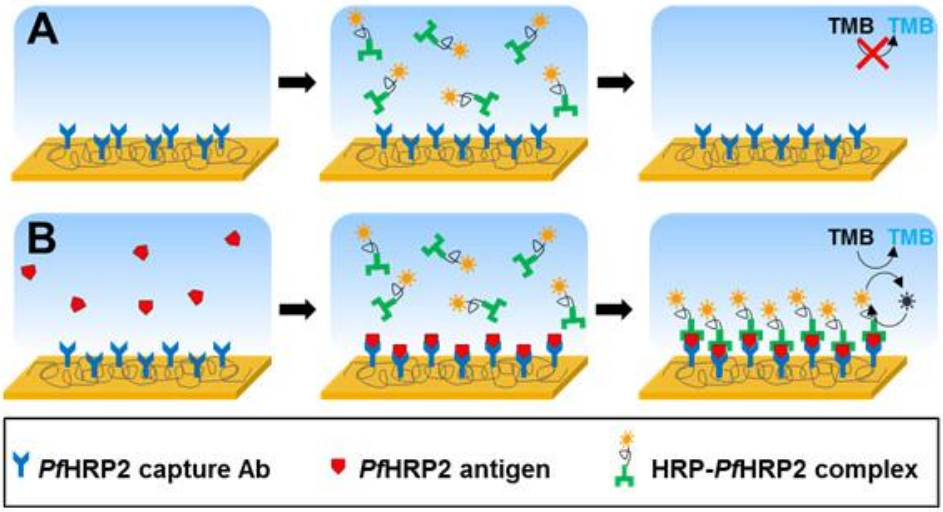

Figure 8. Electrochemical detection scheme in absence (A) and presence (B) of HRP-2 antigen [49]

Wang et al. [50] demonstrated non-invasive detection of HE4 (human epididymis protein 4), a biomarker for ovarian cancer, by a strategy that combines the microchip sandwich ELISA and cell phone/CCD-camera-based colorimetric measurement. Concentration of HE4 in serum has been shown to be correlated with the clinical status of ovarian cancer, but Wang and his co-workers used urine as a sample. Their microchips were made of PMMA and double-sided adhesive film. More applications of polymer-based devices are mentioned in Tab.2.

Table 2: Recent applications of smartphones and polymer-based microfluidic devices

Material of the chip	Application	Ref
PDMS	Smartphone-based fluorescence microscope for direct monitoring of organ-on-a-chip (analysis of toxicants effects on nephrons)	[51]
PDMS	Antibody based mycotoxins detection, smartphone used for colorimetry, ImageJ processing	[52]
PDMS	Viscosity measurement, smartphone used for data acquisition and analysis only	[53]
PDMS	Portable blood cell counter with smartphone readout, smartphone only reads the data, the counter is operated by Arduino driver	[54]

Table 2 Continued

PDMS	Zika, Dengue, Chikungunya virus detection based on real-time reverse-transcription loop-mediated isothermal amplification on card, real-time fluorescence imaging is analysed by smartphone	[55]
PDMS	Smartphone operated and controlled liquid handling system (liquid manipulation, pneumatic valves etc.)	[56]
Polymer, integrated device	Subpicomolar determination of blood markers using immunoassay on nanoscale polymer brush with 3D printed optomechanical interface for the smartphone allowing sensitive fluorescence detection	[57]
Polymer chip	In-terrain PCR with solar thermal system with temperatures controlled by the smartphone	[58]
Polymer cuvette	Construction of a smartphone-based spectrometer with LED light source	[59]
Custom made, PMMA	Determination of herpes simplex virus type 2 using smartphone adapter called “smart-cup”, smartphone used for fluorescence detection using optical filters, MATLAB software used for color analysis	[60]
Polyethylenenaphtalate	Impedimetric array in polymer microfluidic cartridge for detection of D-dimer to aid diagnosis of deep vein thrombosis and pulmonary embolism	[61]
PET/EVA, PVC	A multilayer polymer-film inertial microfluidic device for high throughput concentration of microalgae and trace tumor cells from large background fluids	[62]
PLA, PMMA	An FDM-based 3D-printed microfluidic device with an integrated PMMA optical window for determination of nitrite, total proteins, nitric oxide and microorganism visualization	[63]

Glass, other materials and fluid manipulation via smartphone

Kim et al. [64] developed a microfluidic DNA sensor with mobile interface that employed the transduction of DNA hybridization into a readily detectable electric signal by means of a conformational change of DNA stem-loop structure. To demonstrate the use of this platform, Kim’s group used it for the detection of 100 nM E. coli sequences and the automatic interpretation and mapping of the detection results via a mobile application. The sensor implements two working electrodes from gold, which contain an immobilized DNA probe in the stem-loop structure holding methylene blue in proximity to the electrode surface, which enables the efficient electron transfer with high reduction peak current and a platinum counter electrode, and a platinum reference electrode. The specific target binding emerges when

the target sequence is introduced into the chamber the induced conformational change (stem-loop opening) generates a large decrease in the reduction peak current of methylene blue. After the detection of pathogen, the sensor is reset by washing with distilled water for future use. Table 3 provides more recent applications of smartphones and microfluidic devices made from glass and other materials. Fluid manipulation is also possible by using the connection of a smartphone and microfluidics. For example Zeng et al. [65] developed a compact digital microfluidic analysis platform controlled by a smartphone. The smartphone had two roles there; the first one was to manipulate the droplets on an electro-wetting-on-dielectric microfluidic device and it also served as a reader. Through the smartphone camera, the chemiluminescence signals of the H_2O_2-luminol-HRP system was quantified. From RGB channel only the blue channel was chosen for data analysis, because it was found that the luminescence of H_2O_2-luminol-HRP stimulates it the most. However, there were some problems in the data processing, for example, droplet mixing, non-uniformity, which results in non-uniform distribution of the pixel intensity along with the time and location, spherical shape of droplets (also causes the non-uniformity), etc. Therefore, they calculated the average pixel intensity of the top thousand brightest pixels to represent the concentration of the sample. During the data analysis, the binary image shows the pixels chosen to count in the object image. Their device was used for testing the concentration from1 to 20 mM of H_2O_2. Different approach for fluid manipulation was used by Wixforth [66]. He used surface acoustic waves to actuate and process the smallest amounts of fluid on a piezoelectric chip. Chemical modification of the chip surface created virtual wells and tubes to confine the liquids. The acoustic waves strongly interacted with the liquid and built up an acoustic radiation pressure in the fluid and this pressure was basically the origin for the SAW mediated internal streaming in the fluid as well as for the actuation of small droplets as a whole. The lithographically modulated wetting properties of the surface defined the fluidic network and allowed to create the fluidic tracks, containers, or functional elements.

Table 3: Recent applications of smartphones and microfluidic devices made from other materials

Material of the chip	Application	Ref
ITO, glass	Determination of E. coli and Salmonella sp. based on immunoagglutionation, sample droplets irradiated using fluorescence light, fluorescence intensity measured by ImageJ software	[67]
Polymer support with screen-printed electrode	Development of smartphone-based platforms for POC diagnostics applications, application for detection of dopamine	[68]
PP film with screen-printed electrode	Driving/reading interface for electrochemical sensor test incorporated directly into the smartphone, analysis of uric acid in blood	[69]
Digital microfluidic chip	Electrowetting on dielectrics device on 3D printed support operated by smartphone	[70]
3D-printed chip	Determination of urine proteins by 3D printed microfluidic components using colorimetric analysis, smartphone is used for colorimetric detection	[71]
Glass	Smartphone-based microscopic determination of B. anthracis spores after incubation	[72]
Glass	Microfluidic device prepared by FLICE method, which enables the separation of the two components by exploiting the non-mixing properties of laminar flow.	[73]

2.4 HOW TO PRODUCE MICROFLUDIC DEVICES?

Lithography

Conventional lithography is a process in which the substrate is covered with a layer of photoresist (radiation-sensitive polymer) and locally exposed to radiation to make it patterned. The radiation modifies the structure of the polymer chain to change its solubility and after the resist is developed in a suitable solution and desired pattern is created onto the substrate. Conventional lithography includes photolithography and beam lithography.

Photolithography

Photolithography has substrate covered with a photoresist and a photomask above it. The photomask has dark and bright parts, these parts allow the radiation to penetrate the selected areas. There are two types of photoresist in photolithography positive and negative. With the positive photoresist the bright parts of the mask, where the radiation gets through cause the resist material become soluble in a developer solution and the dark parts are protected and remain on the substrate after development in the appropriate solution. In a negative photoresist the exposed parts become insoluble and unexposed parts are soluble in the developer solution. The exposing radiation is typically ultraviolet. The main disadvantages of this method are high costs and permanent features of photomasking. Principle of photolithography shows Figure 9. [74–76]

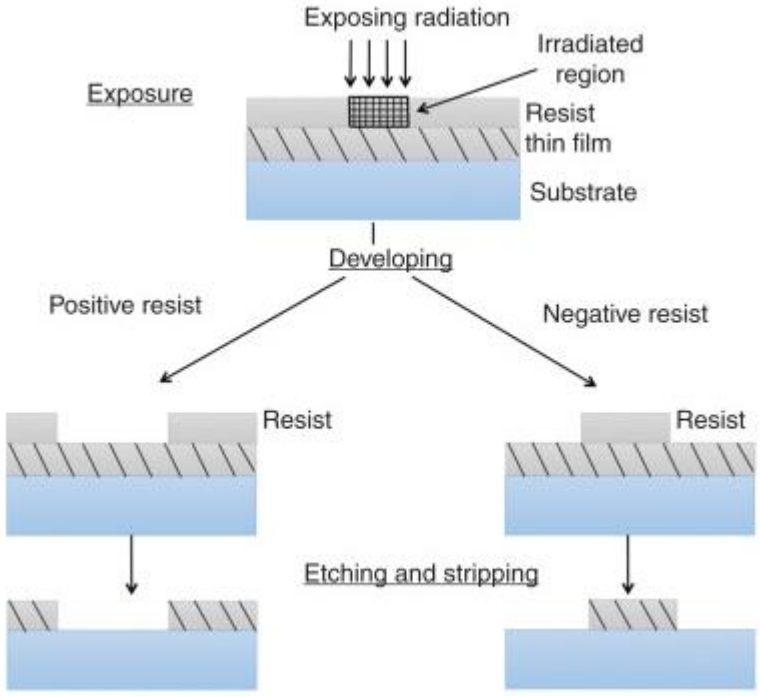

Figure 9. Photolithography principle [76]

Soft lithography

Soft lithography was introduced by Whitesides in 1998. Soft lithography includes techniques involving soft polymeric mold such as PDMS replica from an original hard master.

Mold masters are typically fabricated by photolithography. Thus, the mold masters get the stamp pattern, which is transferred on substrate material and creates the channels. Soft photolithographic approaches such as microcontact printing and capillary molding were introduced to fabricate micro/nanopatterns onto a surface or within microfluidic channels. [77–79]

Contact printing generates non-structured, chemically modified surface. It is direct patterning method using an elastomeric stamp prepared by soft lithography. PDMS stamps are soaked in molecular „ink“ and brought into conformal contact with the substrate on which is the ink transferred. Capillary molding is combining a nanoimprint and the use of an elastomeric mold. In this method is patterned PDMS mold placed on a polymer surface and heated over the polymer´s glass transition temperature. Capillary forces the polymer to melt into the void space of the channels formed between the mold and the polymer. Instead of PDMS it is possible to use also solvent-laden polymer or a UV-curable resin followed by solvent evaporation or exposure to UV light, for e.g. polyurethane functionalized with acrylate groups has been introduced to replace PDMS molds for sub-100-nm lithography, which allow the use of capillary molding in the cell biology studies. A graphical representation of both of the above methods is shown in Figure 10 [80–85]

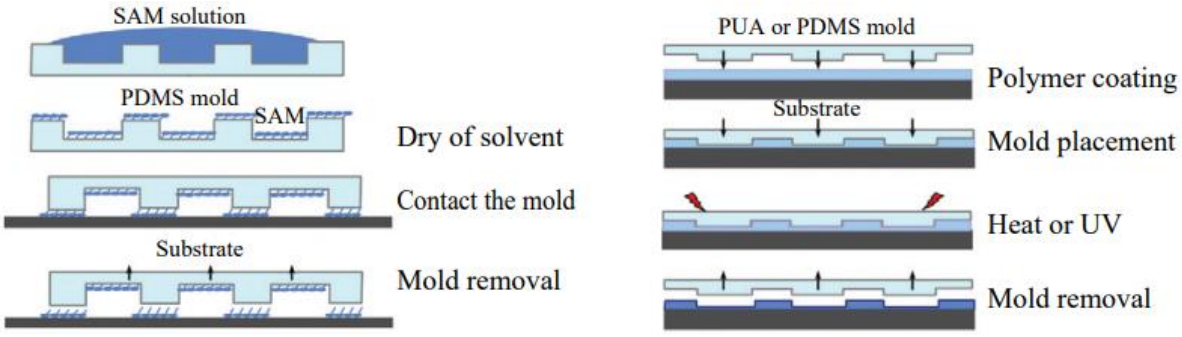

Figure 10. Contact printing (left side), capillary molding (right side)

All lithographic methods prepare mold master, which is forming the channels structure into polymers or other materials used for microfluidic device fabrication. Most common material is previously mentioned PDMS. Prepolymer of PDMS is mixed with curing agent in ratio of 10:1, poured onto mold master and cured over night or at least 4 hours in the oven with temperature about 60°C. After the curing is the PDMS peeled off and final device is sealed

by sticking onto microscope glass or other PDMS layer for example by plasma treatment. This process provides us closed channels with desired structure. Main advantage of lithography is its capability to provide straight, narrow and well-defined channels, but at the same time its main disadvantage is the high cost of production, given by the need of a clean room. What is more, photolithography has limitations due to the potential cytotoxicity of the photoinitiator, the need for specialized equipment, and the difficulty in patterning the surface without modifying the surface topography.[78,86] Therefore, in the next section I would like to focus on more economical production methods.

Lamination

Lamination and laminates are two words which could explain in different ways. Here I would like to describe laminate microfluidic devices, which are fabricated as a stack of layers, independently cut, which are bonded together to form channels. Each layer can provide a two-dimensional flow geometry, which is closed by layers above and below. Basic layered devices consist of three layers: an interface layer, a flow layer and a bottom layer.[87] An example of three-layer device used for microfluidic PCR was fabricated by Jafek et al. [88]. This device consists of two layers of a microscope slide (lower and upper) and a flow layer cut from double-sided tape. Upper microscope slide has ports cut into it, to form closed channel with the acrylic ports glued on as interfaces. This example illustrates many of benefits of layered microfluidic devices: relatively inexpensive materials, simple process steps, rapid fabrication times, well-controlled layer depths etc. [89]

The height of the channel is defined by the thickness of a material which is used, and the width of the channels depends on the cutting method or forming a flow layer. Most common materials used in lamination devices are adhesive transfer tapes, polymer layers (polycarbonate, PMMA and COC) and glass slides. Two most common and straightforward bonding methods are adhesives and thermal bonding. Adhesive layers can be directly the flow layers or adhesive layers between layers of different materials. Adhesive bonding only struggles with high pressures and can be prone to uneven bonding and channels heights. In thermal bonding, temperature close to the glass transition temperature of one or both materials are used, and a force is applied across the surface of the layers. Mostly is used for polycarbonate and polylactic acid. During thermal bonding, the material may warp during heating or subsequent

cooling. [90–92] Some application using lamination for microfluidic fabrication are mentioned in Tab. 4.

Table 4: Recent applications of microfluidic devices made by lamination

Material	Application	Ref
Polyester films	Two polyester films laminated together with xerographic toner in between to create channels	[93]
COC films	Lamination based replication technique for rapid fabrication of sealed microfluidic devices with a 10 cm long linear separation channel.	[94]
Polymer	A microfluidic analyser module constructed of a plurality of channel forming laminate layers that are directly bonded together without adhesive or other possible contaminant source located proximate the flow channels	[95]
Paper and Polyester films	Laminated paper-based analytical device (LPAD)	[96]
Paper and Polyester film	LPAD device for Cotinine Detection in Mouse Serum	[97]

3D Printing

3D printing is a layer-by-layer manufacturing technology, which creates solid three-dimensional objects or structures under precise digital control. The general process uses a 3D digital model, that is sliced into layers and can be created by printer. The most relevant 3D print techniques to microfluidic devices fabrication are fused deposition modeling, multi jet modeling and stereolithography. I will focus only on fused deposition modeling, which will be a part of practical part of this thesis.[98]

Fused deposition modeling (FDM)

Fused deposition modeling is the most common extrusion-based 3D printing technology, which is used by current desktop 3D printers for printing of a variety of biocompatible thermoplastics such as acrylonitrile butadiene styrene (ABS), PLA, polycarbonate, polyamide and polystyrene. This method uses a nozzle, where material is melted, to print different layers of 2D planes on top of each other, where second layer binds to first before cooling. In 2002 Whitesides´ group used an FDM printer to make a mold

for PDMS casting, but FDM is also capable of printing multi-material objects. One of these materials usually serves as sacrificial material.[98–101] For example He et al. demonstrated simple microfluidic device created from novel microfluidic printer, that extruded melted sugar (maltitol) with pneumatic force. Sugar lines were printed on PDMS base layer followed by casting PDMS onto the sugar layer and this process was repeated. After the PDMS was cured, the microfluidic devices were formed and placed in boiling water to dissolve the maltitol pattern.[102] Basically we can say that FDM is used in a similar way as lithography to provide „mold master“ for PDMS casting or as sacrificial material. More application using 3D print are mentioned in Tab. 5.

Table 5: Recent applications of microfluidic devices made by 3D printing

3D print method	Application	Ref
FDM	Capillary valves in the centrifugal microfluidic disc	[103]
FDM	Reaction device for chemical synthesis and purification	[104]
FDM	3D microfluidic created by casting a curable resin (epoxy resin) around sacrificial scaffold (isomalt)	[105]
FDM	Microfluidic immunosensor for three prostate cancer biomarkers detection	[106]
Stereolithographic 3D print	The creation of microchip electrophoresis devices for analysis of medically significant biomarkers related to risk for preterm birth	[107]
FDM	3D-printed microfluidic device for the synthesis of silver and gold nanoparticles	[108]

2.5 HOW IS THE DETECTION DONE IN MICROFLUIDICS?

Detection in microfluidics can implement almost every common analytical method, i.e. optical detection, electrochemical detection (amperometric, voltammetric, potentiometric, and conductivity detection), mass spectrometry, nuclear magnetic resonance.[109–115] But also enzyme reactions can be part of detection in connection with conventional detection methods. ELISA and other immunoassays in connection with chemiluminescent detection were published in several papers e.g. Heyries et al. use it for allergen-specific antibodies detection.[116] Other unconventional methods appearing in conjunction with microfluidic devices are thermal lens microscope detection (type of photothermal spectroscopy), thermal

detection, acoustic wave detection and LEDs.[117–119] LED or mostly OLED (organic light emitting diode/device) detection was firstly published in the early 1960´s by M. Pope [120] and W. Helfrich [121] et al.). For experimental part of this thesis I will pay more attention only to optical, colorimetric and already mentioned branch - smartphone based detection in chapter 2.3, describing materials used in microfluidic fabrication.

Optical detection

Optical detection methods in microfluidic devices have two approaches: it can be done „off-chip“ or „on-chip“. Optical detection, especially spectrometric detection is common not only in microfluidics as there is a wide range of applications (absorbance, fluorescence and chemiluminescence). In order to utilize these into micron-sized detection areas, the use of pinholes at focus points along the optical path or optical fiber is commonly called as “off-chip approach”. The „on-chip approach“ on the other hand, is integrating not only fluidic elements, but also electrical or other type of elements into microfluidic platform, which should be able to perform all chemical functions and detection in a single device.[122] Some specific examples of optical detection are shown in Tab.6.

Table 6: Recent applications of optical detection in microfluidics

Approach	Optical detection	Analyte	LOD	Ref
Off-chip	Absorbance	Thiourea	167 µM	[123]
Off-chip	Reflectance	Ca(II) in urine	2.68 x 10^{-5} M	[124]
Off-chip	Fluorescence	pH	2-11	[125]
Off-chip	LIF	Protein	10 µM	[126]
Off-chip	Chemiluminescence	Catechol, dopamine	~ µM	[127]
Off-chip	Bioluminescence	ATP	~ µM	[128]
Off-chip	Electrochemiluminescence	Lincomycin, proline in urine	9 µM	[129,130]
On-chip	Absorbance	Crystal violet	0.54 µM	[131]
On-chip	Fluorescence	Fluorescein carboxyfluorescein	1 µM	[132]
On-chip	Chemiluminescence	Hydrogen peroxide	10 µM	[133]
On-chip	Refractive index	Air, methanol, isopropanol	-	[134]

Colorimetric detection

Colorimetric assays are reactions leading to a change of color due to enzymatic or chemical interaction between reagents and the analyte. Color intensity of reaction spot could be judged by naked eye, but many factors can affect it like visual perception of color, lighting and the color difference between for e.g. wet paper after reaction and a dry printed color on a label from previous calibration step.[135] To quantify the results more accurately the digitalization of resulting color is necessary. Many devices can be used for this purpose. For example the hand-held optical colorimeter, which measure transmission of light through paper[136], cell phone cameras[137] or a scanners, where the device picture is converted to CMYK format and using image processing software the change in intensity of the color is quantified and related to the concentration of analyte.[138] Cell phone cameras have the benefit of immediate picture sharing after the on-site testing. However there are still interpretation errors coming from experimental conditions such as lighting, sensitivity of the color measurement device, background color of the paper substrate.[137]

Colorimetric detection is ideal for simple, semi-quantitative answer or when a yes or no judgement is needed. Colorimetry usually focuses on a linear response over the range of interest observation. The dynamic range is limited by the amount of reagents and sample present, so colorimetric reaction needs to be faster than the rate of evaporation. The dynamic range can be improved by keeping the device incubated in a humidity chamber, which reduce the evaporation.[138]

2.6 WHAT IS LAB-IN-A-SYRINGE?

The lab-in-a-syringe (LIS) technology represents an innovative methodology in the field of biosensing based on the concept of lateral- or vertical-flow assays and microfluidic paper-based devices (μPADs). The concept of lateral flow assay (LFA) tests are well-known from the common pregnancy tests, aimed at detection of the presence of hCG (Human chorionic gonadotropin). In vertical-flow assay (VFA) tests, is the flow of liquid vertical compared to the LFA. They are formed by the layers stacked on the top of each other, similarly as layered or “origami” μPADs.[139,140] The LIS concept can only serve for sample pre-treatment such as extraction, filtration before conventional analytical analysis or involve the entire analysis inside the syringe. The main problem of the LIS concept lays in the detection.

During the LFA tests is the detection pad easily visible for users. But in LIS tests, user must open the cartridge, holding the detection pad. This represents the crucial practical problem in case of analysing potentially harmful or infectious samples. Table 7 shows that syringes (LIS) can be used can be used for both sample pre-treatment and whole analysis.

Table 7: Recent applications of use of sample pre-treatment in a syringe and LIS

Approach	Analyte	LOD	Ref
Cartridge connected to a syringe	10 allergens	1 ng/mL	[141]
Two cartridges connected together with one connected to a syringe	IcG PSA	1 ng/mL 1.9 ng/mL	[142]
Two cellulose acetate membranes sandwiched between reusable plastic filter holders connected to a disposable syringe	D- and L-Alanine	0.77mM	[143]
Ordered mesoporous silica fiber packed between two cotton layers in the hub of syringe (lab-in-a-syringe SPE)	plasma peptide enrichment, on-site peptidomic sampling	-	[144]
Cloud point extraction on a LIS and spectrophotometric detection	Antimony	-	[145]
The reaction of the metal ion with the chelating reagent, the analyte micro-extraction and transportation of the extractant to the detection system for ETAAS quantification	Metals	5.7 ng/mL	[146]
A porous graphene sorbent coated with titanium(IV)-functionalized polydopamine for selective LIS extraction	Phospoproteins and phosphopeptides	-	[147]

3. AIMS OF THE THESIS

The topic of the doctoral thesis is the study, production and testing of microfluidic devices from various low-cost materials. Particular task included:

- extend the knowledge from MSc research by creating the low-cost test for water analysis from laminating foil and paper,
- extend the knowledge from Ph.D. internship, which included developing the solution of PDMS wafer fabrication from low-cost material,
- apply the acquired knowledge of the production of microfluidic devices in combination with other materials, including the use of syringe,
- testing of fabricated devices on analysis, which could be used in Third World countries,
- studying of application of smartphones in connection with detection.

These goals led to publication output:

- **Lenka Hárendarčíková**, Daniel Baron, Andrea Šebestová, Jan Rozsypal, Jan Petr, True lab-in-a-syringe technology for bioassays. Talanta 174 (2017) 285-288.
- **Lenka Hárendarčíková**, Jan Petr, Fabrication of low-cost polydimethylsiloxane master from laminating foil for isotachophoresis separation on a chip, Instrumentation Science & Technology, 46 (2018) 316-325.
- **Lenka Hárendarčíková**, Jan Petr, Smartphones & microfluidics: Marriage for the future. Electrophoresis 39 (2018) 1319-1328.
- Palacký University Olomouc. A portable low-cost device for a multiparameter chemical analysis. Originators: Jan Petr, Vítězslav Maier, Martin Švidrnoch, **Lenka Hárendarčíková**, Czech utility model 30744, registered at Czech Industrial Property Office Prague on 13th June 2017.
- Palacký University Olomouc. A portable low-cost device for a multiparameter chemical analysis. Originators: Jan Petr, Vítězslav Maier, Martin Švidrnoch, **Lenka Hárendarčíková**, Czech Patent 307 248, registered at Czech Industrial Property Office Prague on 14th of March 2018.

4. EXPERIMENTAL PART

4.1 FABRICATION OF LOW-COST POLYDIMETHYLSILOXANE MASTER

Most of the data presented in this section have been also published in ref. [148]

Chemicals

3-Ton Clear epoxy adhesive was purchased from Alteco (Broumov, Czech Republic). Methanol was purchased from Sigma-Aldrich (Prague, Czech Republic). Polydimethylsiloxane and curing agent (Sylgard 184) were purchased from ELCHEMCo (Zruč nad Sázavou, Czech Republic). Bromphenol Blue, amaranth red, hydrochlorid acid, 2-(N-morpholino)ethanesulfonic acid and glycine were of p.a. quality and purchased from Sigma-Aldrich (Steinheinm, Germany). Deionized water of 18.2 MΩ cm was produced from MilliQ (Millipore USA).

Master fabrication

The Fellowes office laminator Mars A3 and laminating pouches Fellowes (Itasca, IL, USA) were laminated together. After the bonding, carved for Petri dish size (the final device was casted inside). Carved foils were washed with distilled water, methanol and dried using compressed air. Clean wafer base – foil circles - were cut in desired structure with fabricated knife fixed in the desktop corner cutter (Warrior, Taichung. This knife was made from two technical trapezoidal knives with triangle shaped blades, which were precisely glued together by epoxy adhesive. The glued „double-knife“ was cured overnight. Scheme of the fabricated knife with size data is shown in Fig. 11.

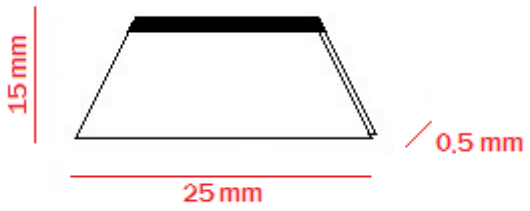

Figure 11. Scheme of fabricated knife with dimensions

The fixation of the cutter ensures the use of the same cutting pressure and better reproducibility of the depth of the channels (the carved structures could not penetrate the whole foil depth). After the cutting process, the cleaning procedure was repeated. In the final product, the microfluidic channels arise between those cuts. The dimensions of the wafer were analysed using the contact profiler Form Talysurf Series 2 (Leicester, UK).

Curing of PDMS and device preparation

Prepolymer of PDMS and curing agent were mixed in the ratio of 10:1, stirred properly and then degassed in vacuum. [149] The time of degassing depends on the size of the wafer. The process is finished, when no bubbles are visible on the surface of polydimexyl siloxane layer. Petri dish was covered with aluminium foil to protect the glass from prepolymer mixture. Next, the foil master was fixed into Petri dish before pouring of PDMS because of density and weight of the foil, it did not stay at the bottom, but floated on the prepolymer layer. Fixture was done by spreading a layer of prepolymer mixture on the aluminium foil (or master from the bottom) and cured in the oven at 70°C for 10-20min. The layer of PDMS held the master and afterwards, the prepolymer was poured onto the master and cured at 60°C over night.

The cured PDMS slap with defined structures was peeled off from the master, the reservoirs were punched with biopsy punch 1.5 mm size (from Miltex, USA) and cut for single device. The cut devices and microscope slides were washed with methanol and dried with compressed air. Manipulation with both parts was performed with tweezer to protect it from dust and other impurities. Final device was sealed with air plasma Zepto Rie (from Diener Electronic, Germany), which stuck the microscope slide and PDMS layer together. The pressure of the air used in plasma cleaner for bonding was fifty mbar for thirty seconds. Whole process is drawn in Fig. 12.

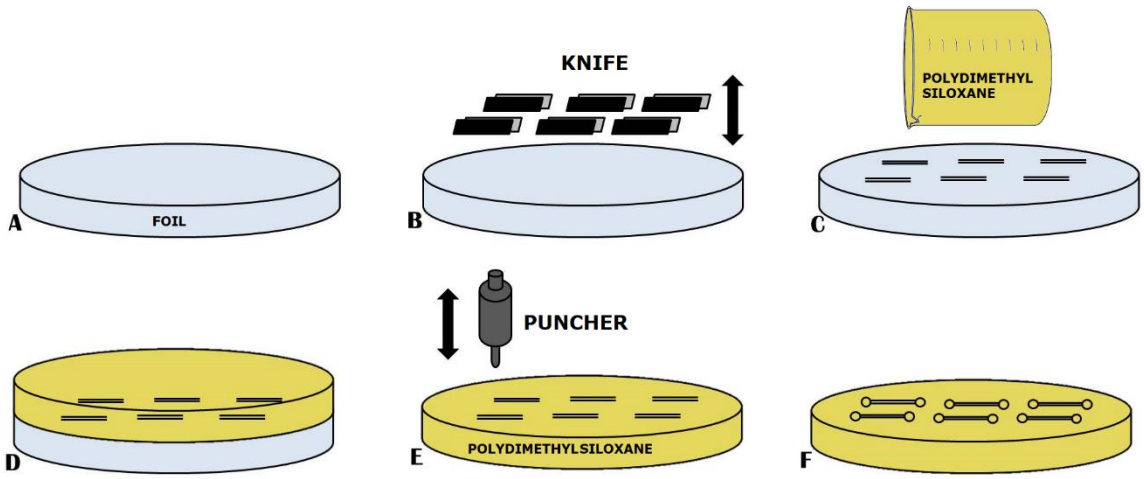

Figure 12. Scheme of the wafer and PDMS chip fabrication: (A) carved laminating foil, (B) punching of channels with customs made knife, (C) punched foil and prepolymer pouring, (D) PDMS curing, (E) reservoirs punching and (F) punched PDMS before cutting for single devices.

Isotachophoresis (ITP)

Isotachophoretic separation of two organic dyes – bromphenol blue and amaranth red was studied to prove the concept of low-cost master fabrication. In isotachophoresis, the sample is injected between two electrolytes. One electrolyte contained the fastest co-ion with the highest mobility (leading electrolyte) and second contained the slowest co-ion with the lowest mobility (terminating electrolyte). After application of an electric potential charged analytes are separated based on ionic mobility (this factor tells how fast an ion migrates through an electric field), during separation process the analytes create zones and in the final steady state these zones are arranged in order of their mobility and migrate with same velocity. Migration time is dependent on the mobility, capillary length and electric current. Sharp boundaries between neighbouring zones are typical for ITP as a result of jumps in voltage between them. This principle shows Fig. 17 in part „results and discussion“. [150]

Sample preparation

The solution of 10 mmol L^{-1} hydrochloric acid served as the leading electrolyte and 10 mmol L^{-1} 2-(N-morpholino)ethanesulfonic acid served as terminating electrolyte. The pH of both electrolytes was modified to 4.8 by glycine. The sample composed of mixture of 100 µL of bromphenol blue (10^{-3} mol L^{-1} in deionized water), 100 µL of amaranth red (10^{-3} mol L^{-1} in deionized water) and 100 µL of the leading electrolyte.

Results and discussion

Wafer fabrication

After previous combination of lamination foil and paper or membrane, there was idea of using laminating foil for production of the whole microfluidic device, by stacking layer of foil between two, which were sealing them into final device. Advantage of this principle was that part inside, which were creating shape of the channels, could be cut in different shapes, from thicker or thinner foil. Overall, we can prepare a diverse range of microfluidic channels. But the main disadvantage is that foils are glued (by thermobonding in laminating machine), so for e.g. if you try to fill the foil with hot agarose to prepare channels for gel electrophoresis the structure of the channel will be destroyed. Figure 13 shows early studies of the behavior of laminating foil devices on a larger scale. All devices were filled with agarose (1% m/v) colored with brilliant blue dye (Merck, Czech Republic) for better visualization of leakage after hot filling.

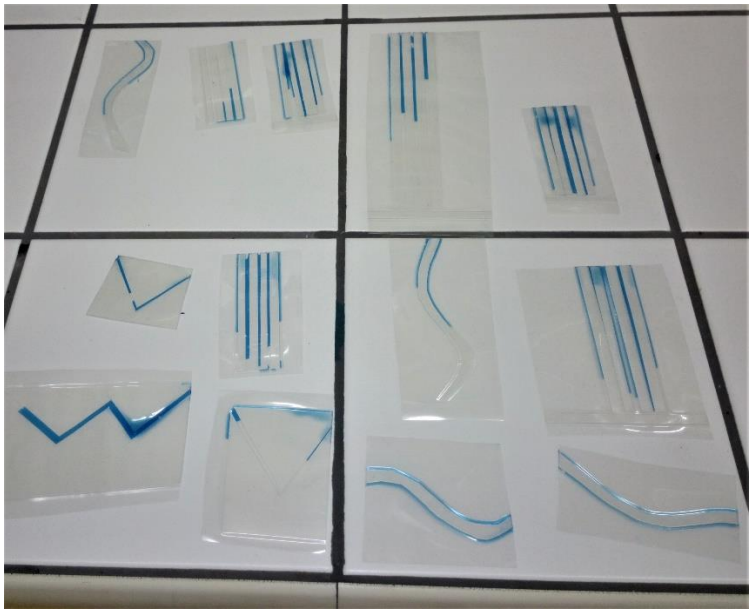

Figure 13. Different shape of channels in foil filled with colored agarose gel

After several similar results from other experiments, the decision was made, that it would be better to use lamination foil only as a base for PDMS device. Lamination foils were chosen as a base material for masters preparation because of their inertness, smoothness (which is necessary for the foil to be non-stick with cured polydimethylsiloxane) and flexibility. We have studied five foils widths: 80, 100, 125, 175 and 250 µm. The grooves were cut onto all foils and then the PDMS chip was fabricated, as described above. Afterwards the grooves were analysed under the microscope and it was found that the best repeatability of the groove structure was obtained using 100 µm foil width. This is probably due to the strength of the laminated foil and the pressure needed for the channel preparation.

The next parameter tested was the use of the fixed knives in the desktop corner cutter or the use of fine precise hand cutting. The hand cutting was very repeatable, when one person repeated the procedure, the relative standard deviation of the channel width was about 5%, n=10. But the reproduction capabilities rapidly dropped, when two people repeated the procedure. The relative standard deviation of the channel width was over 80%, each n=10. The foils are tough enough to hold the desired shape but also not tough enough to be punched to desired structure. Although, it seemed that the desktop corner cutter is not fit for fixing the knives. However, we found that the key step in the procedure is cutting, hence, the knives were fixed in the desktop corner cutter under the small angle to ensure the cutting the foil, not

punching. This led to the reproducible formation of grooves with the similar precision as was for one person hand cutting.

The knives, as described before, form two straight grooves with triangle cross-section (typical for cutting) having width of 90 ± 8 µm and depth of 51 ± 6 µm. The distance between them is 344 ± 4 µm as shown Figure 14. All values are calculated from 5 different grooves, each measured in three positions. Average value with standard deviation is displayed.

Within the fabrication of PDMS chips, the cut grooves (negative depth from the base) formed PDMS images (positive depth from the base; „hills“), which were then bonded on the glass slide forming a channel between both „hills“. The channel width was still quite precise, 326 ± 41 µm. Although, the channel precision and resolution cannot compete with traditional lithographic procedures but it can allow analytical procedures where precision is not the key parameter and it is significantly cheaper, e.g., using such devices as sample pre-treatment tools or for specific separations; e.g., where the channels are filled with agarose or polyacrylamide gels. [151]

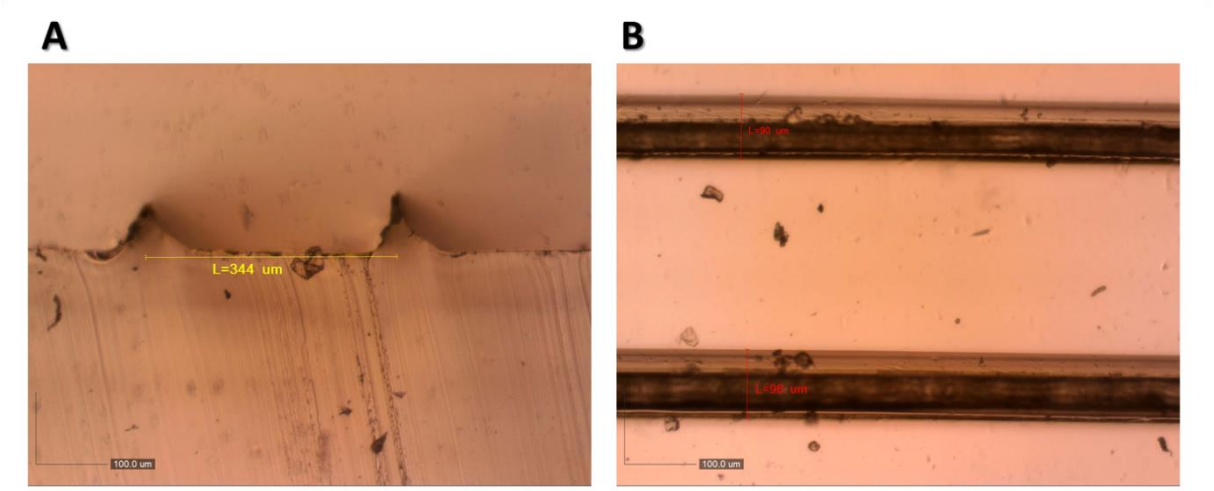

Figure 14. Fabricated channels: (A) cross-section of the cut channels, (B) top-view of the cut channels

The width of the channel can be easily varied by changing the space between the glued knives, for example by putting an extra slice of metal between them or by using smaller or thinner knives. Picture of the whole device with smaller channels is shown in Fig.15.

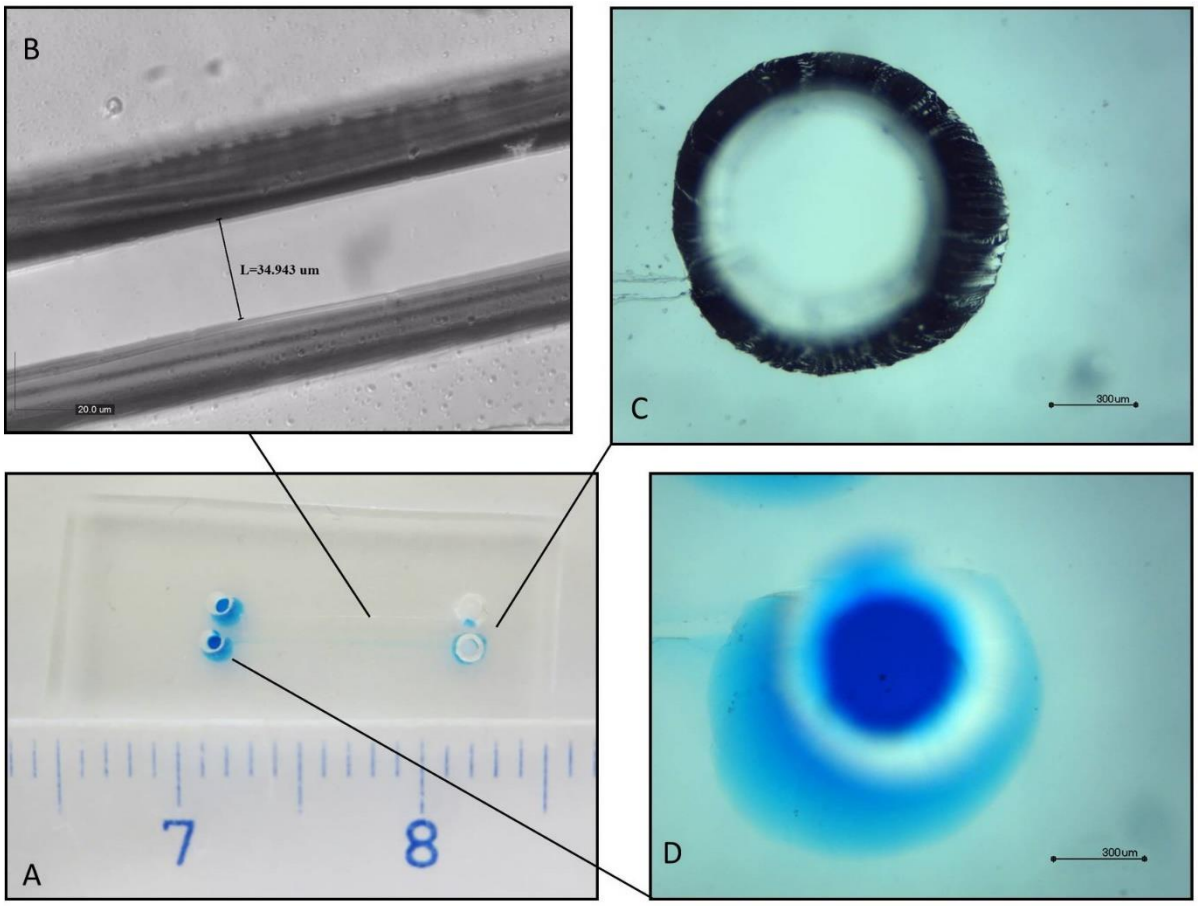

Figure 15. (A) Complete device (photo took with Canon PowerShot S110, **(B)** magnified channel (FLIM, fluorescence microscope, IntracoMicro), **(C and D)** magnified image of empty reservoir and filled one with brilliant blue dye (Microscope VSM 52, IntracoMicro)

3D view of channel with different dimensions of used knife, we can see thanks to image from 3D microscope VHX-6000 (Keyence, Japan) on Fig. 16.

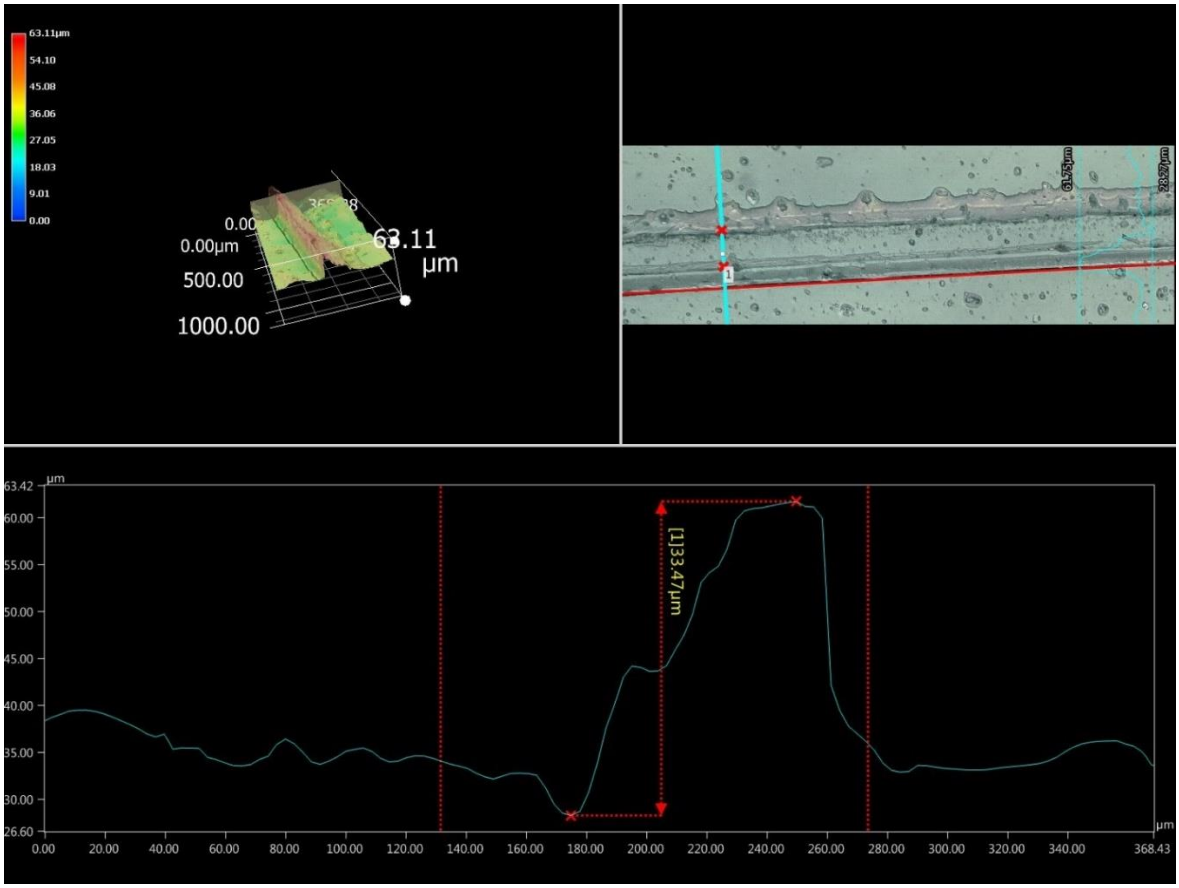

Figure 16. 3D view of microfluidic channel fabricated with our solution

Isotachophoretic separation

To prove the concept of our device we chose model separation of two organic dyes. Isotachophoresis system was established according to the previously published conditions. [152,153] The analysis was running in the straight channel device with the channel length of 2.5 cm with 10 mmol L^{-1} HCl as the leading electrolyte and 10 mmol L^{-1} 2-(N-morpholino)ethanesulfonic acid as terminating electrolyte, both at pH 4.8. The whole device was filled with leading electrolyte after polydimethylsiloxane bonding to avoid the loss of channel hydrophilicity. The subsequent isotachophoretic separation process is described step by step in Figure 17. At first, the leading electrolyte was replaced with the sample solution and the electric field of 20 V cm^{-1} was applied for 45 s. After sample loading, the reservoir was emptied and rest of the sample solution was washed out by the terminating electrolyte. Afterwards, the reservoir was filled with terminating electrolyte and the electric field

of 20 V cm^{-1} was applied again to start the separation process. After a few seconds, the sample movement was apparent, around a minute after voltage applying, the separation process began and after approx. 3 min, the separation of two organic anionic dyes was achieved as shown Figure 18.

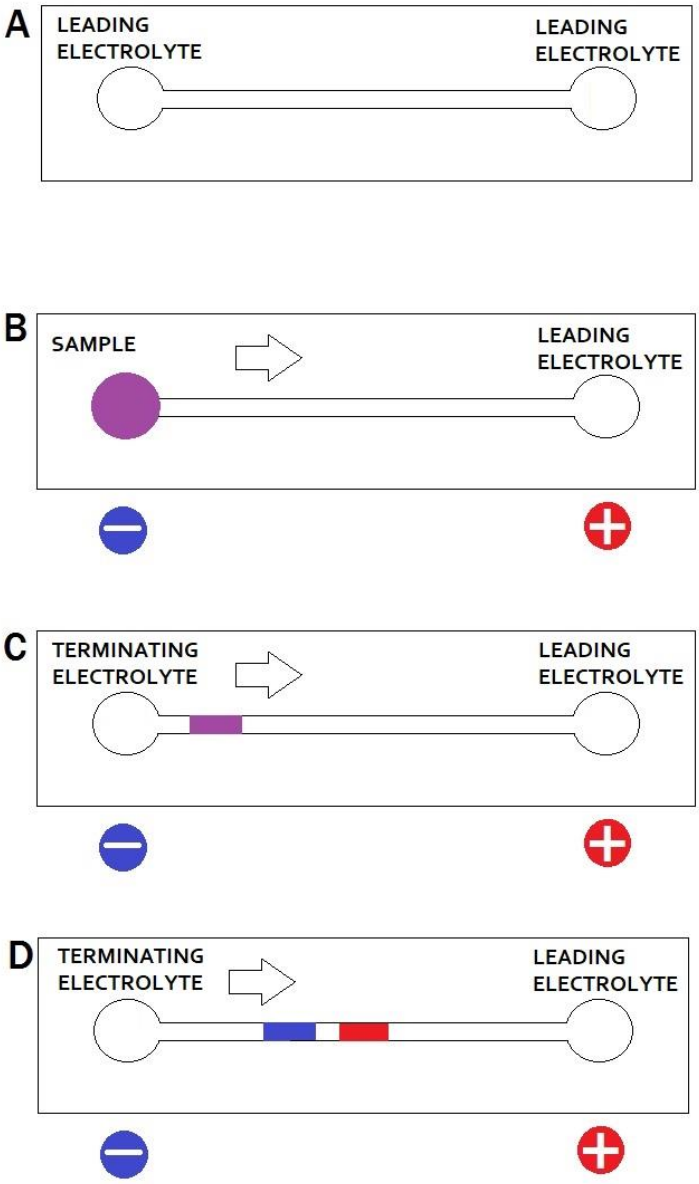

Figure 17. Scheme of isotachophoretic process: (a) Device filled with leading electrolyte, (b) reservoir filled with sample and sample loading, (c) reservoir filled with terminating electrolyte, and (d) separation

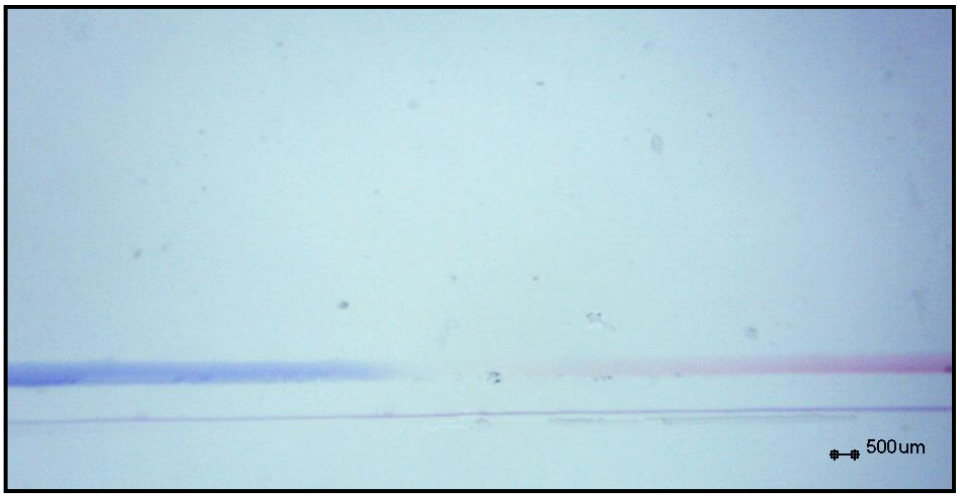

Figure 18. Example of isotachophoretic separation of bromphenol blue and amaranth red.

Conclusion

The new master fabrication process allows us to produce foil master with channel dimensions of dozens of microns. The main advantage is the possibility to quickly modify the design and shape to perform the desired analysis even in low-cost laboratories or studies, where a cleanroom and expensive instruments are not available. The preparation time is also incomparable, because preparation of the master (excluding knife fabrication) takes just couple of minutes, and besides cleaning there is no additional step necessary. The net costs of wafer preparation were approx. 200USD, which cover office laminator, box of laminating pouches (100 pieces) and customs made knife. At this price, we could prepare 600 masters because from one foil we were able to prepare six masters of Petri dish size. It means, the costs of one wafer was around 0.34 USD. There are also advantages such as fabrication time and simple channel design changes. The advantages of our wafer, compared with the one fabricated with photolithography are summarized in Table 8, all prices are approximate.

Table 8: Summarization of photolithography and foil wafer processes

Parameter	Photolithographic wafer	Foil wafer
price of one wafer	**32 USD**	**0.34 USD**
chromium mask for photolithography	**319 USD**	**0 USD**
time of fabrication / amount of fabricated wafers	**120 min / 5**	**15 min / 6**
operating costs of fabricating (2 hours)	**85 USD**	**0.23 USD**
possibility to change the mask?		**YES**
need of cleanroom	**YES**	**NO**

4.2 PORTABLE LOW-COST DEVICE FOR MULTI-PARAMETER CHEMICAL ANALYSIS – AQUACHIP

Most of the data presented in this section have been also published in ref. [154,155]

Chemicals

Reagents for pH 3-10 and 9-13 and sulfuric acid were purchased from Fluka (Prague, Czech Republic). Nessler´s reagent, sulfanilamide, hydrochloric acid, N-(1-naphthyl)ethylenediamine dihydrochloride, chromotropic acid disodium salt dihydrate, calconcarboxylic acid, 1,10-phenantroline monohydrate, ammonium thiocyanate, hydrazine hydrate, sodium chloride, sodium fluoride, sodium nitride, silver nitrate, ammonium hydroxide solution, calcium chloride, Iron(III) chloride hexahydrate, Iron(II) chloride tetrahydrate, sodium sulfate anhydrous, potassium iodide, natrium hydroxide, phosphoric acid, zirconium(IV) chloride and poly(ethylene glycol) 3000 were purchased from Sigma-Aldrich (Prague, Czech Republic). Methanol was purchased from VWR Prolabo chemicals (Rožnov pod Radhoštěm, Czech Republic). Schwarzenbach buffer was purchased from Mikrochem Trade (Pezinok, Slovakia). Ammonium molybdenane tetrahydrate was purchased from Lach-Ner (Neratovice, Czech Republic). Deionized water of 18.2 MΩ cm was produced from MilliQ (Millipore USA).

Sample preparation

Basic ion solution was prepared in volumetric flasks of 10mL volume by dissolving solid salts or diluting the concentrated solution to concentration 2 mol L^{-1}. This basic solution was used for preparation of a set of calibration solutions. Calibration was done in range from 0.25 mol L^{-1} to 2 mol L^{-1}.

The pH reagents and Nessler´s reagent have been prepared by the manufacturer and used directly for testing. For determination of nitrites mixture of solutions in ratio of 1:1 was used. For the first solution 0.5g sulfanilamide and 5 ml of HCl (32%) were dissolved in deionized water and made up to 50 ml in a volumetric flask. Second solution was 0,05 g of N-(1-naphthyl)ethylenediamine dihydrochloride dissolved in deionized water and made up to 50 ml in a volumetric flask. The nitrates reagent consists of 1 g of chromotropic acid disodium salt dihydrate dissolved in deionized water (50 ml volumetric flask).

For determination of calcium ions was used mixture in ration of 1:1. One part of this mixture was 250 mg of calconcarboxylic acid diluted in 50ml of methanol and second part was Schwarzenbach buffer. 0,2 % solution of 1,10-phenantroline monohydrate in 0,1 mol L^{-1} HCl was used for ferrous ions. For ferric ions 0,1 mol L^{-1} solution of ammonium thiocyanate in deionized water was used. [156–159]

Aquachip fabrication and use

Aquachip consists of three basic parts – carrier part (see Fig. 19-1), reaction part (Figure 19-8) and a part, which ensures the transport of the sample to the reaction zones (see Fig. 19-16). On a filter paper (Whatman Grade 1) used as a base for reaction cells is printed the structure of reaction part. Wax printer (Xerox ColorQube) is used to print the hydrophobic pattern (see Fig. 19-8) onto filter paper. Since the printed wax layer is only on the surface and not through the entire paper structure, it is necessary to press the paper and place it in the oven or iron it between a non-sticky layer such as baking paper. After the formation of wax barriers, the paper is cut into three reaction parts (see Fig. 19-9, 19-10 and 19-11).

Carrier part consists of lower covering layer (see Fig. 19-2) which is formed from credit card size laminating foil, in which instructions for working with the device can be laminated from the back side of the device (we were not using it in our experiments). Furthermore, only a half of the laminating pouche with the adhesive layer (see Fig. 19-3) is used to create upper cover piece (see Fig. 19-4), having a cut shape corresponding to other parts of the device. Under this layer name of device and instructions of maximum line for immersion of the device in the analysed sample can be placed (see Fig. 19-26). The notch on Figure 19-6 corresponding to the insertion of the reaction portion and the notch (see Fig. 19-7) corresponding to insertion of the sample transport pads (see Fig. 19-17 and 19-18) to the reaction zones.

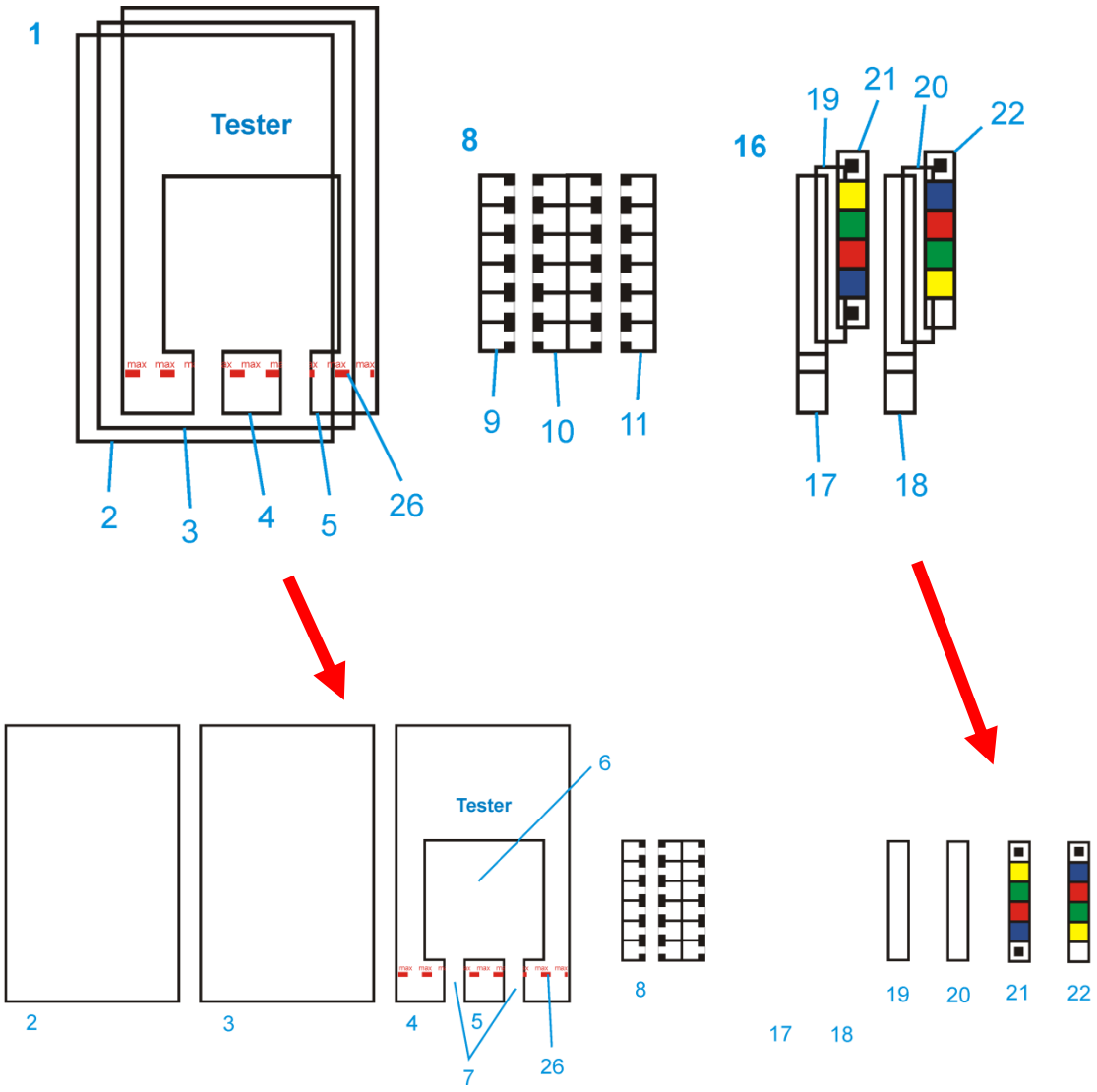

Figure 19. Individual parts of Aquachip

Figure 20 shows further details of structure of the reaction portion. Reaction portion is formed from square reaction zones (see Fig. 20-12) having a thin edge (see Fig. 20-13) on three sides and a wide edge (see Fig. 20-14) on one side with a gap (see Fig. 20-15). To perform the analysis, specific reagent for a particular test parameter is applied to the square space, to fill the entire square space. The hydrophobic wax barriers ensure no liquid transfer between the reaction zones. Also, the amount of liquid applied must not exceed the content of the square, because then it would contaminate other parts of the device through the gaps. Reagents are applied according to their characteristics, some need to be dried immediately, some may partially break the barrier and therefore it is not possible to apply them to the entire area of the square. The liquid sample to be analysed flows through the gap to the reaction site

(square with reagent). The reaction part is aligned and glued onto the cut-out on the upper cover part of the support part of the device.

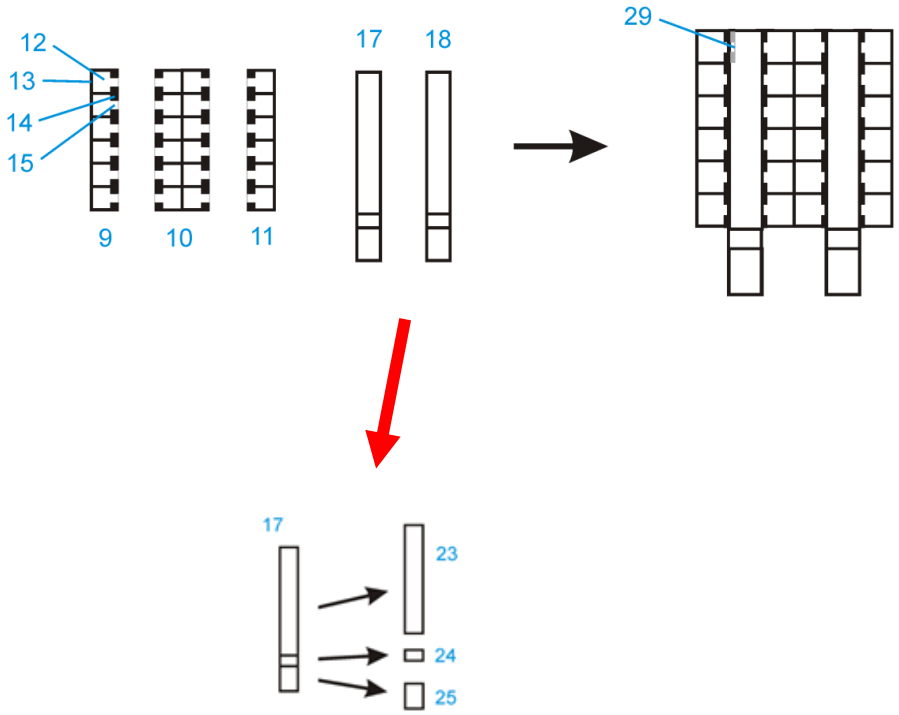

Figure 20. Detail of the reaction zone and transport pads

The „sample transport part“ is composed of three aligned parts which are on the apparatus in two identical copies, because the samples are transported simultaneously by two routes. Specifically, there are following parts: the hydrophilic layers leading the sample to the reaction zones and dispensing the sample (see Fig. 19-17 and 19-18), adhesive layers (see Fig. 19-19 and 19-20) and evaluation layers (see Fig. 19-21 and 19-22). A hydrophilic layer is a cellulose fiber membrane (CFSP203000 from Millipore, Labicom Czech Republic) used to absorb samples in test strips. This layer may be either non-divided or divided into multiple portions. Figure 20-23 serves as a transport of the sample to individual reaction zones. The middle portion Figure 20-24 modifies the sample and removes the impurities from the sample. It can be impregnated with agents causing such changes. Its location is below the beginning of the reaction portion of the device. The same can be performed in other parts of the device. However, in the case of chemical modification of the sample, including for e.g. pH adjustment, it must be carried out at a point where no other contamination occurs in the reaction zones. The lower part (see Fig. 20-25) is used to suck the sample. Its size,

respectively length is determined by the maximum immersion of the device in the sample. If the intermediate portion would be below the maximum immersion limit of the device, there is a risk that the sample will not be completely modified by the reagents contained in the intermediate portion. The hydrophilic layer is flushed and glued into the cut-out (see Fig. 19-7) on the upper cover part of the support part of the device. It is essential to align this layer with the reaction part of the device on the reaction part, where it covers part, mostly half of the width of the wider side of the reaction zones. This will ideally overlap the portion that transports the sample to the reaction zones and entre the reaction zones. If such overlapping did not occur, the analysis would not be fast enough and there would be risk of contamination of the reaction zones with other reagents from the other reaction zones.

The evaluation zones (see Fig. 19-21 and 19-22) consist of paper with printed squares of the same dimensions as the squares of reaction zones. Three outer squares (see Fig. 21-27) define the position of individual reaction zones on the whole tester for evaluation by smartphones or tablets. The middle squares (see Fig. 21-28) are then used to set the color range for the evaluation software. The length of evaluation layer may be the same as the length of reaction portion or in case of divided hydrophilic sample dispensing layer may be extended to the point of maximum immersion of the device, so that the separation of the layer will not perceptible to the user. But evaluation layer can not be longer than maximum immersion site, because the printed text or colors would be washed away and would contaminate the sample.

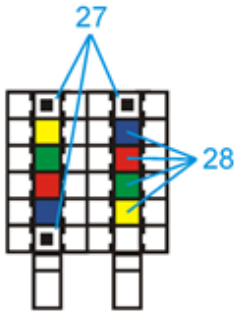

Figure 21. Detail of evaluation zones

Software

The WAXAN evaluation software (for Android platform) is capable of following actions: automatically detects the reaction field of the chip, marks it and cuts the reaction field to avoid the detection of a „black border“, that would make analysis difficult. Then it evaluates the color of the captured pixels, converts them to HSV format (hue, saturation, value), and compares them to the predefined range directly in the software and displays the value of the parameter (e.g. concentration of analyte).

The software does not calibrate the ambient light intensity, it automatically triggers illumination through the telephone before capturing the image (photo). This creates relatively the same illumination conditions.

Results and discussion

At the early stages, before the Aquachip design we have started by choosing the right reagents for our ion analytes. The idea of smartphone detection was already in our mind, so we were focusing on reactions with significant color change, which will be easy for detection. At first, we were trying our reaction with above mentioned reagents on a drip plate (see Fig. 23A and 23B). Only difference here is in determination of nitrates. The first was used zirconium chloride solution. The product of such reaction is a white precipitate.

The white precipitate is possible to use for example on black filtration paper, but it is impossible to judge the concentration of analyte because the color intensity is not changing with different concentration of analyte and also the color of filtration paper is not stable through whole surface. Reaction on filtration paper shows Fig. 22.

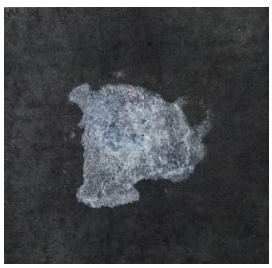

Figure 22. Reaction of zirconyl(IV) chloride with nitrates (concentration 2 mol L^{-1}).

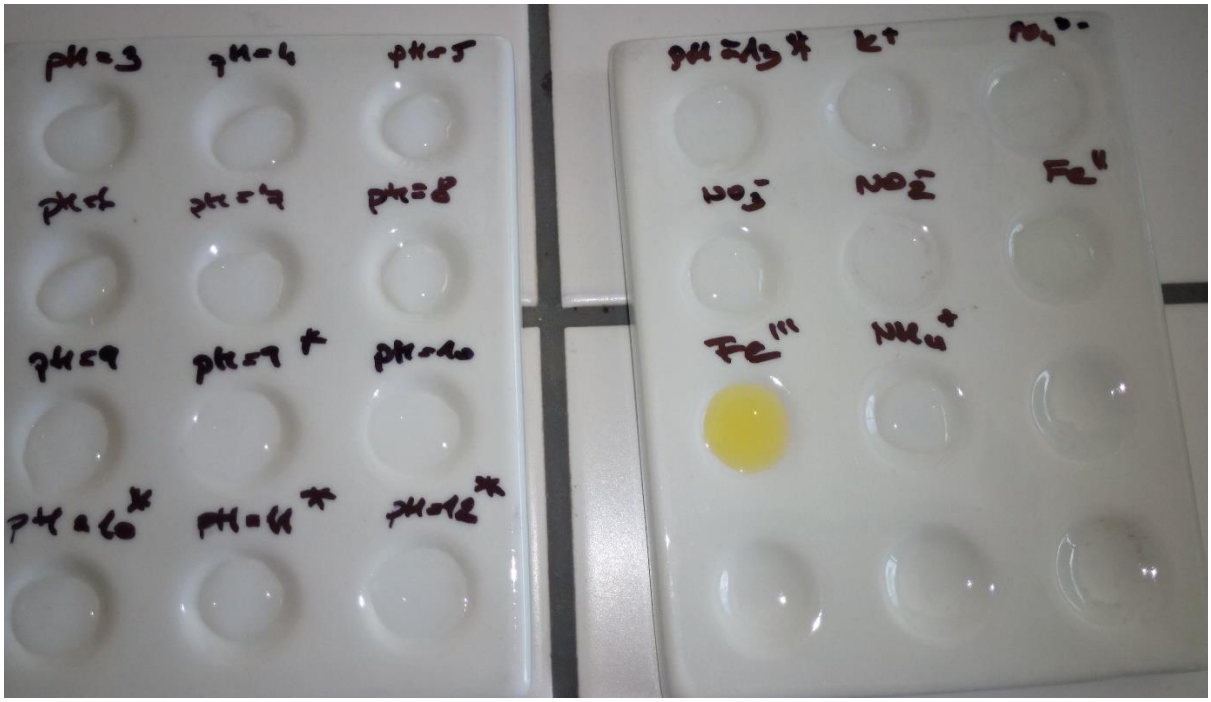

Figure 23A. Drip plate with ion and pH samples before reaction

Figure 23B. Drip plate with ion and pH samples after reaction with appropriate reagents (pH with star mark means use of pH reagent with range 9-13)

Following step was to select the appropriate material and chip arrangement. The material serving as the substrate for the reaction is the most essential part of the whole apparatus. There must be no changes, especially on the surface, due to reactions. The first we considered thicker materials to be able to use a larger sample volume for the reaction itself. First, we tested the plate for liquid chromatography (aluminium foil with anchored stationary phase/slurry). This solution has two major problems; first we are not able to stop or control liquid flow only in „reaction zones“. We tried to scratch the grid, but then the stationary phase became crumbled. Other issue is reaction of ammonium ions with Nessler´s reagent, which destroy stationary phase. Both problems are shown in Figure 24.

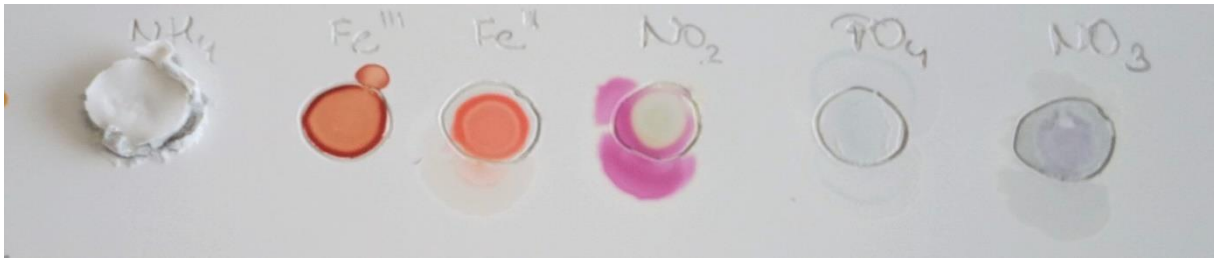

Figure 24. The leakage of reactions and releasing of stationary phase in reaction of Nessler´s reagent with ammonium ions

Because we wanted to support the speed of sample flow, another tested material was cellulose fiber membrane (CFSP203000 from Millipore). Significant advantage is the sample flow rate, but the reaction itself may not be visible on the membrane surface due to its thickness (for example see Fig. 25 shows like 3 different pH values are not possible to recognize in comparison with colors on drip plate), which is necessary for subsequent evaluation by the software on the smartphone. Unlike conventional filter paper, which were eventually used for the reaction zone, we are not able to create a wax barrier through the entire membrane layer, resulting in the reaction zones suffering from leakage. At the end we decided to use filtration paper and wax barrier, as already described above. We have tested different sizes and shapes of the channels in whole device, the division of the reaction zones similar to „QR code“ is ideal to be processed by the software.

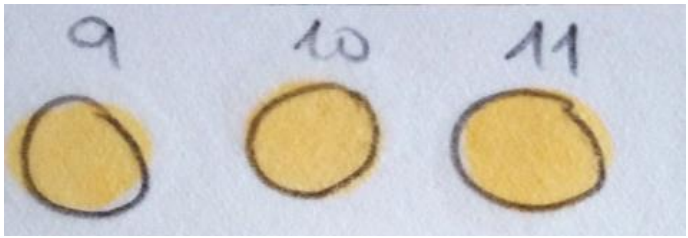

Figure 25. pH 9-11 reaction on cellulose fiber membrane

We also wanted to use the knowledge gained from previous research concerning the implementation of laminating foil, which was meant to isolate the whole analysis from the environment. But when the whole device was sealed, it suffered from leakage and reaction mixing around cellulose fiber membrane which served for sample transport to the reaction zones. In addition, we did not want the reaction zone to pass through the laminator due to possible thermal degradation of the reagent, thus laminating foil was used as carrier part.

Within the prototype of a portable low-cost device for multi-parameter chemical analysis, individual positions were occupied by reaction tests for the analysis of pH, ammonium, calcium, nitrate, nitrite, phosphate and ferrous and ferrous ions. Positions are listed in Table 10 and reaction part division shows Figure 26.

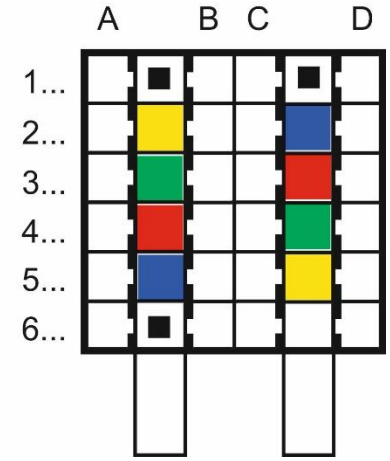

Figure 26. The division of reaction part

Table 10: Position of tests on the chip

Position	Test
1A	pH 3 – 10
1B	NH_4^+
1D	pH 9 – 13
3A	NO_2^-
3C	Ca^{2+}
3D	NO_3^-
5A	Fe^{2+}
5B	PO_4^{3-}
5D	Fe^{3+}

For test preparation 0,5 µL of reagent is always applied per reaction site. In case of mixed reagents, 0,5 µL of mixture is applied to the reaction site (all reagents are mentioned above, together with preparation processes). Afterwards the reaction part is dried under a stream of hot air using a hair dryer. Final device is then immersed in water sample containing analytes and after reaction, picture is captured via smartphone camera and reaction and analytes concentration is evaluated. How the real device looks like shows Figure 27.

Figure 27. Real AquaChip photos from both sides

The evaluation of concentration is based on comparison with calibration solution of each ion consisting of concentration range from 0.25 mol L^{-1} to 2 mol L^{-1} and the result color of their reaction with reagents in reaction zones, also the color of background was captured

for the same purpose. From all these reactions HSV values were collected as shows Figure 28 (only one example), which our IT member implemented into software algorithm. Analytical parameters of AquaChip shows Table 11.

Table 11: Analytical parameters of AquaChip

Analyte	Concentration range	Accuracy*
pH	3 – 13	Δ pH 3
NH_4^+	130 – 1200 mg L^{-1}	30 mg/L
NO_2^-	140 – 920 mg L^{-1}	30 mg/L
NO_3^-	190 – 930 mg L^{-1}	40 mg/L
Ca^{2+}	200 – 800 mg L^{-1}	40 mg/L
Fe^{2+}	170 – 2790 mg L^{-1}	35 mg/L
Fe^{3+}	140 – 1400 mg L^{-1}	30 mg/L
PO_4^{3-}	240 – 2850 mg L^{-1}	50 mg/L

* Accuracy depends on software calibration, generally in the order of 20 - 30%

Already mentioned above the software does not calibrate the ambient light intensity, only triggers illumination over the telephone. However, this will vary with different phones, as well as different room lighting can cause an error. The next step would be to incorporate a comparison with the color scale on the chip (lighting calibration) into the software. It is also necessary to process software inhomogeneities in the color of the zone, which is not currently processed by the software. Homogeneous color distribution also causes problems, which often occurs on chips itself. To create a homogeneous color distribution, the patches are soaked with a 1% PEG 3000 solution in water prior to application of the reagents. However, this slows down the analysis.

id	H	L	S
0	9	157	22
2	39	170	10
3	28	154	20
5	35	152	15
6	39	174	11
8	154	38	221
9	34	157	9
11	159	68	101
12	156	72	142
14	39	161	9
15	159	91	73
17	35	145	13
18	33	160	13
20	155	38	228
21	34	150	10
23	165	78	60
24	157	58	149
26	33	145	10
27	19	104	48
29	35	137	12
30	30	151	12
32	33	139	10
33	34	134	8
35	35	127	11
19	2	77	215
16	0	82	180
13	69	72	128
22	65	38	195
25	118	38	162
10	116	50	152
7	31	160	116
28	29	79	232

Figure 28. Example of HSV values obtained from NH_4^+reaction

Our AquaChip solution with smartphone evaluation can serve for e.g. as fast on-site water tester in the field. But this research was only proof-of-concept. We are not able to move much further with our university equipment. Above mentioned reagents were used only as proof-of-concept reagents. Theoretically, other reagents that may provide better analytical parameters (LOD, LOQ, linearity, accuracy) can be used to test the parameters. Also, there are only 9 positions of reaction field occupied out of 24 total positions, so it is possible to extend the test with other analysed parameters. We have also tried determination of some other ions including heavy metals, results are shown in Table 12. So further product direction is possible in the following points:

- Development and testing of better chemical reactions leading to color changes - literature describes reactions reaching better detection limits, respectively in general better analytical parameters, than those used for proof-of-concept - however, these reactions contains specific reagents (including nanomaterial conjugates) that are not commercially/readily available and their costs are high (hence they have not been used in proof-of-concept project), their real potential at reactions on paper is also a question.

- Testing of other reactions for other parameters that could be applied to the "paper tester".

- Development of own tester so that its production price is further reduced.

- Software development for thorough color analysis including small changes and environmental effects (lighting, etc.)

Table 12: Other tested reactions

Analyte	Parameters
Ni^{2+}	30 – 2350 mg/L; error 10 mg/L
Pb^{2+}	100 – 6220 mg/L; error 25 mg/L
Cd^{2+}	60 – 4500 mg/L; error 15 mg/L
Hg^{2+}	100 – 4000 mg/L; error 20 mg/L

Conclusion

The new AquaChip design connects classic paper microfluidic with smartphone detection. The main advantage of the design is the possibility to determine any analyte for which we are able to anchor the reagent onto the filter paper to provide a color reaction. We proved the functionality of our device with basic water analysis/analytes with good analytical parameters, also keeping the production costs low and possibility of the outside-of-the-laboratory use, following our main idea set at the very beginning. There is only the need of storing the finished tests in an airtight container, free from light and moisture. We believe that the AquaChip idea can be further developed from the point of view of the device itself or the software with much wider use.

4.2 TRUE LAB-IN-A-SYRINGE TECHNOLOGY FOR BIOASSAYS

Most of the data presented in this section have been also published in ref. [160]

Chemicals and materials

Standards and reagents, dimethylglyoxime, sodium fluoride, acetic acid, ammonium hydroxide, 1,10-phenanthroline, iron(II) chloride and nickel(II) nitrate hexahydrate, were purchased from Sigma-Aldrich (St. Louis, MO). Methanol for HPLC was purchased from Merck (Darmstadt, Germany). The LIS concept was tested using a model reaction of Ni(II) determination according to the previous works [161,162]. The reagent was prepared as 60 mM solution of dimethylglyoxime in methanol. The blocking agent was prepared as a 1:1 mixture of 0.5 M sodium fluoride and 6.3 M acetic acid (pH 4.5). Finally, the ammonium hydroxide solution (pH 9.5) was used as the reagent buffer. Fe(II) assays was used to study the effect of blocking. Here, 1,10-phenanthroline at concentration 8mg/mL in 6M acetate buffer (pH 4.5) was used as the reagent.

Syringes 2 mL with Luer connection were purchased from Chirana (Stará Turá, Slovakia), Xcut Docrafts puncher with 9.5 mm circle knife was purchased from Artea Trading (Ostrava, Czech Republic), and cellulose fiber sample pads CFSP203000 Millipore were purchased from Labicom (Olomouc, Czech Republic).

Tester fabrication

Two test types were prepared according to the detection part arrangement used in the test. First, the pads were prepared as follows: Millipore cellulose fiber sample pads were cut with the Xcut puncher to rounded pads with diameter of 9.5 mm. The diameter had to be chosen according to the diameter of the syringe barrel (9.0mm our syringes from Chirana). Then the pads were inserted into the syringe, where they formed filtration, conjugation and flow parts of the device. It should be noted that using the same diameters of rounded pads and the syringe barrel (e.g. 9.0 mm pads and 9.0 mm barrel, or 9.5 mm barrel as used in Braun syringes, for 9.5 mm pads) did not fit well. Same pads formed also the detection part in the first type of the test. In second type, detection part was formed by folding rectangular cut pad (27 mm length and 8 mm height) from Millipore cellulose fiber sample into the syringe barrel (see Fig. 30). The pads for conjugation and detection where inserted into conjugation solution or reaction

solution and dried with hairdryer before inserting into the syringe with tweezers. Pads were one-by-one punched to its final form by inserting the plunger to the syringe. The rectangular detection part was rolled up and inserted into the syringe barrel, again by using tweezers. The number of parts for each part of the test depended on the user and the test. Step-by-step fabrication process is displayed in detail on Figure 29.

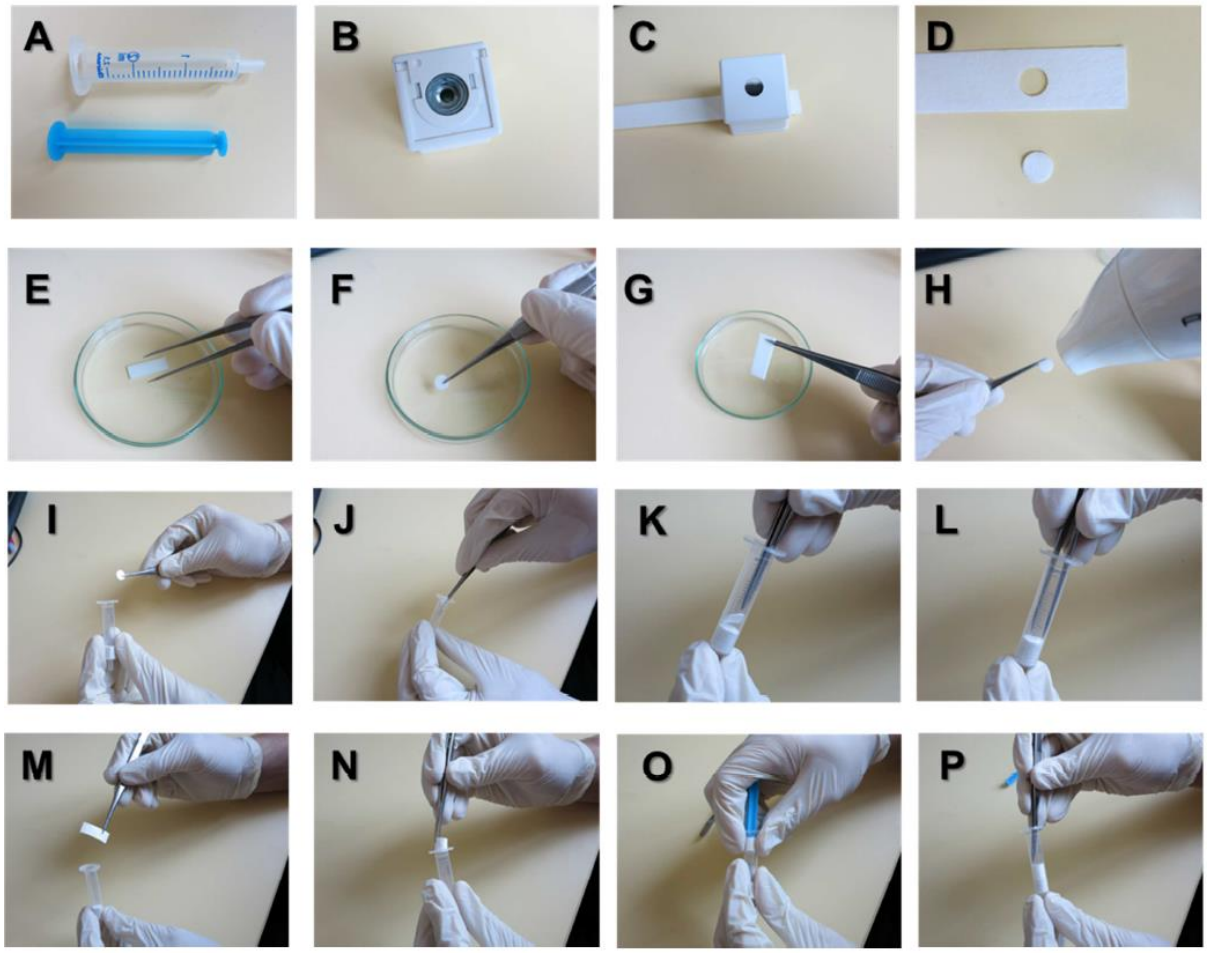

Figure 29. Fabrication process of the lab-in-a-syringe tests

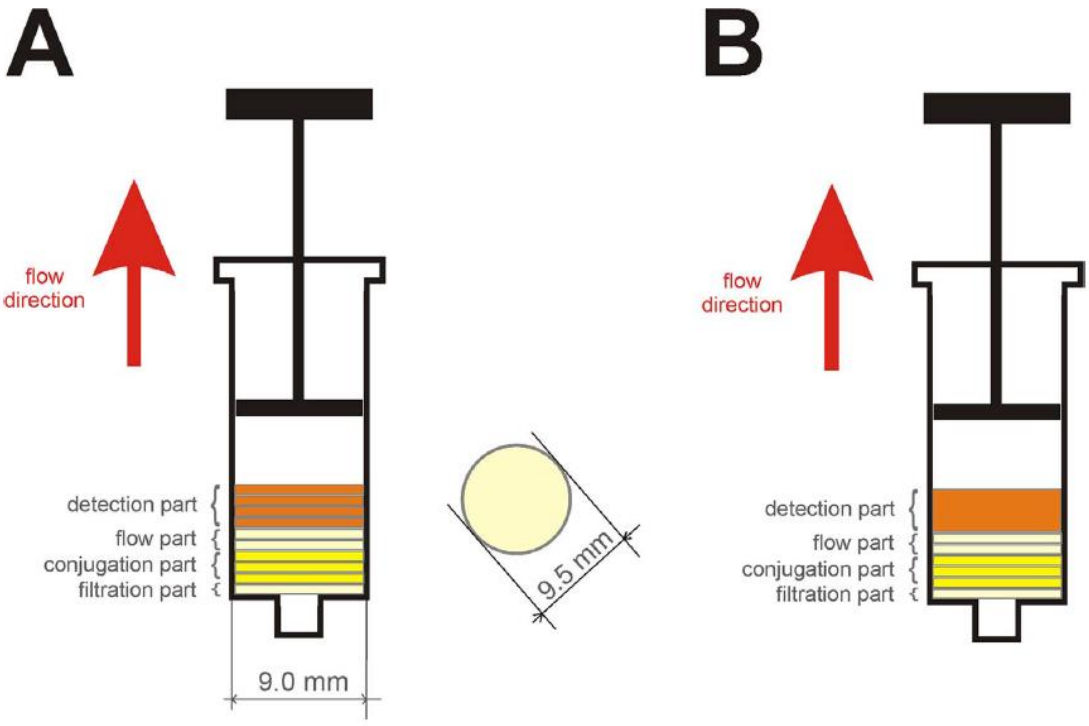

Figure 30. Scheme of the true lab-in-syringe tests. A: LIS test with layered rounded-pads-based detection part, B: LIS test with the rectangular detection part

Detection and processing

After the reaction pads changed its color, we took a photo of the syringes using standard camera Canon PowerShot S110. The image was then transferred into Corel Photo-Paint X4 software. The mask tool was used to pick the colored region and the histogram was then shown. The average value with the standard deviation value was used for calibration. The Ni(II) calibration was done as function of the color intensity calculated as 255 minus the average value on the concentration of the sample.

For testing the blocking agent two limits were determined. First, the intensity of the blank solution, which contained all reagents without Fe(II) was measured, second, the intensity of the non-blocked Fe(II) sample was measured as the 100% intensity. All the other measured intensities were recalculated to percentage using subtraction of the intensity of the blank solution.

Results and discussion

The main problem of the LIS concept in comparison to the classical LFA tests (lateral-flow assays for e.g. common pregnancy test) lays in the detection of the assays.[163] In LFA tests, the detection pad is easily visible for users, but in LIS test, users must open the cartridge which holds the detection pad. This can be dangerous when analysing potentially harmful or infectious samples. We have designed a novel concept of LIS tests, where the conjugation and reaction pads were put directly into the syringe barrel. We have also designed two possible arrangements of the detection part where the detection is user friendly: (A) by using "layered" device where the detection part is composed of a number of rounded pads (five in our case), that increase the thickness of the detection part that needs to be visually analysed, or (B) by using rectangular detection part with an appropriate thickness that is inserted into the barrel (see Fig. 30).

We used classical Ni(II) reaction test with dimethylglyoxime as the reaction agent and the fluorides in acidic environment to block potential cross-reactive species as a model reaction. However, any kind of conjugation solution can be used instead of the blocking agent and also any kind of reaction agent can be used, including traditional immunoassay-based agents. First, we designed the pre-reaction parts of the true LIS test, this is very important mainly for the real samples, where small particles can adsorb conjugation (or blocking agent) or just simply need to be free of undesirable impurities. We used the insertion of one rounded bare part before any other that serves as a primary filter of the reaction solution. Then the conjugation pads were following layer. The number of these pads depends on the amount of the conjugation (blocking) agent reacted. We used Fe(II) as a model compound that needs to be blocked, so the number of pads inserted into the blocking agent should be in correspondence to the amount of covered (blocked) metal. Fe(II) at 1.0 μg/mL were injected into the syringe through 0-5 conjugation (blocking) pads. Quantification of Fe(II) was performed by using classical reaction with 1,10-phenanthroline as described above (we already know this reaction on Millipore cellulose fiber from previous research/chapter). Figure 31 shows, that the amount of blocked Fe(II) slightly increased from 0 to 3 pads, then it reached the acceptable level of 4 or 5 pads. More than 90% of Fe(II) was blocked, so this leading us to the use of 5 pads in the end. This amount ensures blocking of all cross-reactive compounds.

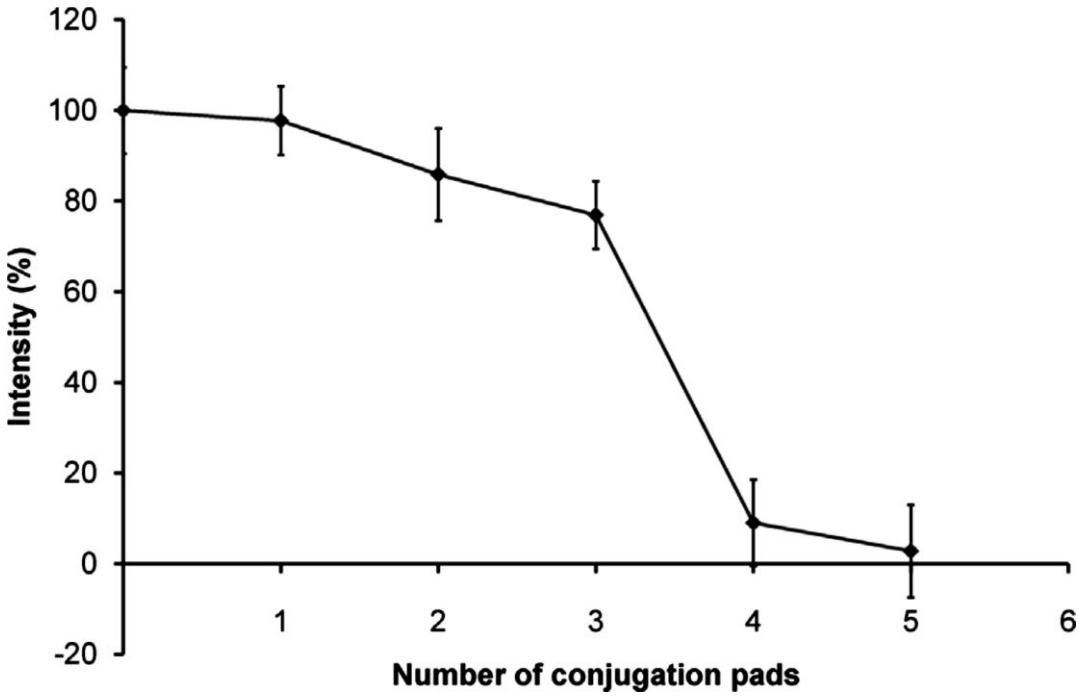

Figure 31. Effect of number of blocking (conjugation) pads on the blocking of Fe(II)

Apart from the main research we studied the effect of insertion of more bare pads between conjugation (blocking) pads and reaction pads. These “flow pads” (as named in Fig. 30) served for the transportation of a sample and for the separation of conjugation and reaction parts. We found that the flow pads were very important for separation of these two tests part, because they prevented leaking of both solutions (also by atmospheric water). In our case, we used three flow pads, as the optimal number. However, flow pads could serve also for separation of potential multiple products and/or products and matrices using paper chromatography principles similarly like in LFA tests. The number of these pads depends on the reaction used in the LIS assay and the associated reagents.

Last aspect of our research was the user-friendliness of the visual detection, which is also the key parameter. We compared two test designs: (A) the layered rounded-pads-based-detection part, and (B) the rectangular detection part. The width of one reaction pad is about 1.5 mm, which makes the test easy to be performed but the visibility is not perfect. Using 5 pads, results in the visible reaction zone having a width of about 7.5 mm, which is fine for common visual detection. Figure 32 shows analysis of different concentrations of Ni(II), clearly showing

the joints of pads being brighter than other places on the pads. But overall evaluation by naked eye estimates the average intensity, by the same principle as the software we used -Corel Photo-Pain. The same test was done with second arrangement using the rectangular detection part with the similar width of 8.0 mm. Results are shown in Figure 33, where can be seen that the suction of the sample into the barrel was more precise in the case of rounded pads-based detection part. This occurred because in rectangular arrangement, the sample liquid could have washed out the precipitate of Ni(II)-dimethylglyoxime complex that hampered the evaluation. Both arrangements provided good linearity in the selected range of concentration with R^2 of 0.998 for rounded-pads detection part and R^2 of 0.931 for rectangular detection part.

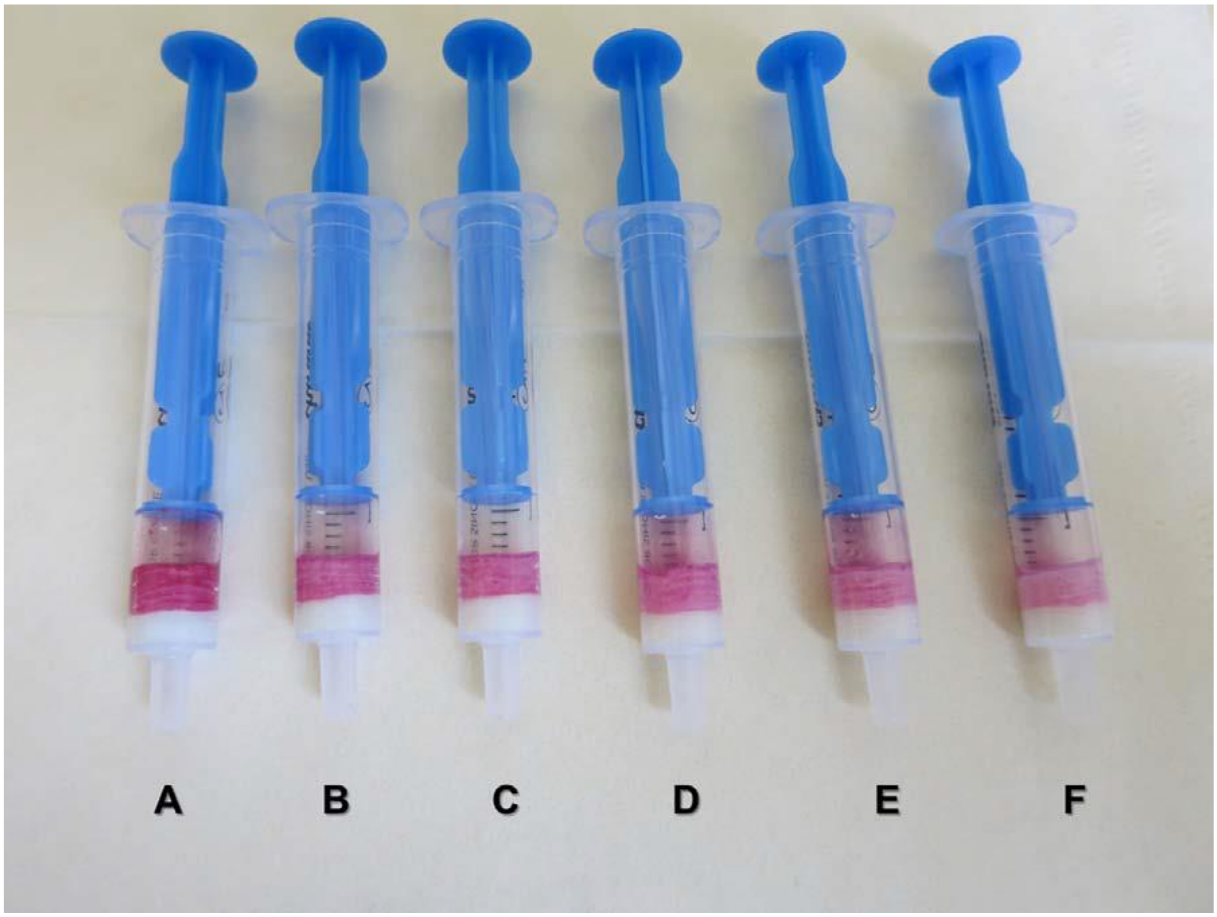

Figure 32. Ni(II) calibration using true LIS test with rounded pads for detection. Ni(II) concentration: A: 0,1 M, B: 0.04 M, C: 0.02 M, D: 0.01 M, E: 0.005M, F: 0.0025M

Figure 33. Photography of Ni(II) calibration using true LIS tests with the rectangular detection part

For user-friendly visual quantification without need of camera, the syringe test can be also enhanced by putting the colored scale onto the barrel. User can easily compare result with this scale, similarly like in pH paper test (see Fig. 34).

Finally, we studied the use of the absorption part over the detection part of the syringe. However, we found that it was not very profitable to use it probably due to the fact that the syringe plunger formed greater flow of the analyzed liquid that could be absorbed by such part.

Figure 34. Syringe barrel with colored scale

Conclusion

In our work we solved a problem of detection in LIS test, by putting all the pads directly into the syringe barrel using more layers to make the results visible for users. It is not necessary to open the cartrige holding the detection part to achieve the detection/quantification. We proved the functionality of our device with determination of Ni(II) levels, but generally the device can be used for analysis including immunoassays because the pads can be divided into conjugation as well as reaction ones. Our true LIS test production costs approx. 2 USD which represents also very low unit price. What is more, there are no specific requirements for storing, as the true LIS tests can be easily stored using classical procedures applied for syringes for medical use. We believe, that the true LIS tests can be very profitable for analytical chemistry in general but they can find use elsewhere.

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LIST OF ABBREVIATIONS

µPADs	microfluidic (micro) paper-based analytical devices
µ-TAS	micro total analysis system
AAS	atomic absorption spectroscopy
BSA	bovine serum albumin
CCD	charge-coupled devices
CE	capillary electrophoresis
CMYK	cyan magenta yellow black
DARPA	Defense Advanced Research Projects Agency
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immuno sorbent assay
FDM	fused deposition modeling
FLICE	femtosecond laser irradiation followed by the chemical etching
GC	gas chromatography
GSV	gray-scale-valuation
hCG	human chorionic gonadotropin
HDL	high-density lipoprotein
HPLC	high-pressure liquid chromatography
HRP-2	histidine-rich protein 2
ICP-MS	inductively coupled plasma mass spectrometry
ITO	indium-tin-oxide
ITP	isotachophoresis
LDL	low-density lipoprotein
LED	light-emitting diode
LFA	lateral flow assay
LIS	lab-in-a-syringe
LOC	lab-on-a-chip
MEMS	micro electromechanical systems
OLED	organic light emitting diode/device

PDMS	polydimethylsiloxane
PET/EVA	polyethylene terephthalate/ethylenevinyl acetate copolymer
PLA	polylactic acid
PMMA	poly(methyl methacrylate)
PVC	polyvinyl chloride
RGB	red, green, blue
TMB	tetramethylbenzidine
UV	utltraviolet
VFA	vertical-flow assay

CURRICULUM VITAE

PERSONAL DATA

Name: Lenka Hárendarčíková

E-mail: lenka.harendarcikova@upol.cz

EDUCATION

- 2014 – present — Palacký University Olomouc, Czech Republic

  Ph.D. programme: Low-cost microfluidic devices for biologically active agent analysis (supervisor: Dr. Jan Petr)

- 2012 – 2014 — Palacký University Olomouc, Czech Republic

  Master´s degree in Analytical Chemistry

  Master´s thesis: Development of miniaturized low-cost tests for clinical and forensic analysis (supervisor: Dr. Jan Petr)

- 2012 – 2014 — Palacký University Olomouc, Czech Republic

  Bacherol´s degree in Chemistry

  Bacherol´s thesis: Using of spectral methods for analysis of art objects (supervisor: Dr. David Milde)

INTERNSHIPS

- 08– 09/2013

  State Veterinary Institute – Department of Foreign Substances, Olomouc, Czech Republic. **Method validation for ICP-OES determination of feed.**

- 03– 05/2015

  THE BIOS LAB-ON-A-CHIP GROUP, University of Twente, Enschede, Netherlands. **Microfludic DNA separation in PDMS device. (s**upervisor: Prof. Dr. J.C.T. Jan Eijkel)

PROJECTS

- Internal grant agency UP Olomouc, IGA_PrF_2015_020, "Matrix effect and its suppression in sample analysis", member of the research team
- Internal grant agency UP Olomouc, IGA_PrF_ 2016_016, "Bioanalysis and analysis of food", member of the research team
- Technology Agency of the Czech Republic, Partial project of TA CR project TG01010080 Support of technology transfer at Palacký University in Olomouc, „Water quality control: cheap and elegant" (POC_0301), member of the research team
- Internal grant agency UP Olomouc, IGA_PrF_2017_020 „Matrix effect and detection limit", member of the research team
- Internal grant agency UP Olomouc, IGA_PrF_2018_027 „Efficient sample processing with complex matrices", member of the research team
- Internal grant agency UP Olomouc, IGA_PrF_2019_028 „Innovative methods of sample analysis with complex matrices", member of the research team
- Ministry of Education, Youth, and Sports of the Czech Republic, NPU LO 1305 "Development of the center of advanced technologies and materials", member of the research team
- Internal grant agency UP Olomouc, IGA_PrF_2020_030 „Modern trends in analysis of samples with complex matrices", member of the research team

EDUCATIONAL ACTIVITY

- 2015/2016 – ACH/ACC (Cvičení z analytické chemie 6 hours/week)
- 2015/2016 – ACH/CHV (Chemie vody 1 hours/week)
- 2016/2017 – ACH/ACC (Cvičení z analytické chemie 6 hours/week)
- 2016/2017 – ACH/KLCH (Klinická analytická chemie, "microfluidic and medicine" 4 hours)

PUBLISHING ACTIVITY

- **Lenka Hárendarčíková**, Daniel Baron, Andrea Šebestová, Jan Rozsypal, Jan Petr, True lab-in-a-syringe technology for bioassays. Talanta 174 (2017) 285-288.

- Tereza Mašková, **Lenka Hárendarčíková**, Jan Petr, Determination of Escherichia coli in urine using a low-cost foil-based microfluidic device. Talanta 170 (2017) 36-40.

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- **Lenka Hárendarčíková**, Jan Petr, Smartphones & microfluidics: Marriage for the future. Electrophoresis 39 (2018) 1319-1328.

- Palacký University Olomouc. A portable low-cost device for a multiparameter chemical analysis. Originators: Jan Petr, Vítězslav Maier, Martin Švidrnoch, **Lenka Hárendarčíková**, Czech utility model 30744, registered at Czech Industrial Property Office Prague on 13th June 2017.

- Palacký University Olomouc. A portable low-cost device for a multiparameter chemical analysis. Originators: Jan Petr, Vítězslav Maier, Martin Švidrnoch, **Lenka Hárendarčíková**, Czech Patent 307 248, registered at Czech Industrial Property Office Prague on 14th of March 2018.

CONFERENCE

- Jan Petr, Daniel Baron, Jana Horská, **Lenka Hárendarčíková**, Pavlína Svobodová, Adam Přibylka, Martin Švidrnoch, Vítězslav Maier, Juraj Ševčík, Co-author of the lecture: Capillary Electrophoresis of Nanoparticles: Present and Future Tasks. 4th Annual RCPTM Conference - Devět Skal, 13 - 14 October 2014

- **Lenka Hárendarčíková**, Jan Petr, Poster: Preparation of low-cost microfluidic devices based on filter and bakery papers by using an office laminator. International Interdisciplinary Meeting on Bioanalysis CECE 2014 - Brno, 20 – 22 October 2014

- Martins Rucins, Daniel Baron, **Lenka Hárendarčíková**, Aiva Plotniece, Jan Petr, Poster: Determination of some hormone antagonists by micellar electrokinetic chromatography. International Interdisciplinary Meeting on Bioanalysis CECE 2015 - Brno, 21-23 September 2015

- **Lenka Hárendarčíková**, Jan Petr, Poster: Fabrication of low-cost polydimethylsiloxane master from laminating foil. Advanced in Chromatography and Electrophoresis & Chiranal 2016, Olomouc, 6-9 June 2016

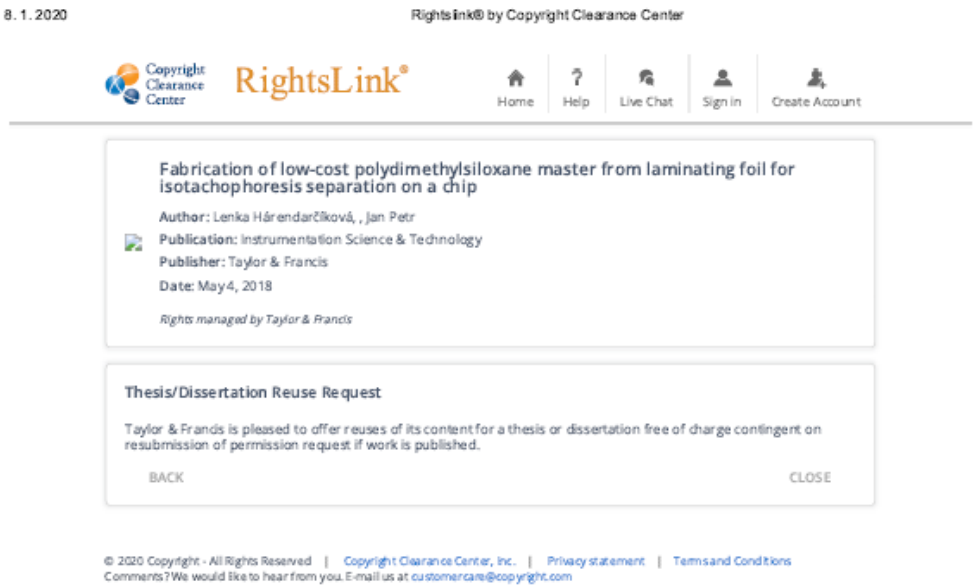
- **Lenka Hárendarčíková**, Jan Petr, Vítězslav Maier, Martin Švidrnoch, Poster: Portable low-cost device for multiparametric water chemical analysis. Advances in Chromatography and Electrophoresis and Chiranal 2018, Olomouc, 29th January – 1st February 2018

ANOTHER_ACTIVITIES

- Reviewer of bachelor theses (Pavlína Svobodová, Petra Hubáčková).
- Organizer and regional secretary of the chemistry olympiad (2014 - 2017)
- Organization and participation of popularization events (e.g. Veletrh vědy a výzkumu, Noc vědců, Den otevřených dveří etc.) and school excursions.
- Master Thesis Consultant (Alena Šustková - Development of microfluidic device using 3D print and its testing for forensic purposes)

APPENDICES

Appendix A. Taylor & Francis permission of reuse of "Fabrication of low-cost polydimethylsiloxane master from laminating foil for isotachophoresis separation on a chip" content in doctoral thesis



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True lab-in-a-syringe technology for bioassays

Author: Lenka Hárendarčíková,Daniel Baron,Andrea Šebestová,Jan Rozsypal,Jan Petr

Publication: Talanta

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PALACKÝ UNIVERSITY IN OLOMOUC

Faculty of Science

Department of Analytical Chemistry

Low-cost microfluidic systems for the analysis of biologically active substances

SUMMARY OF THE DOCTORAL THESIS

Author: Mgr. Lenka Hárendarčíková

Field of study: Analytical Chemistry

Supervisor: doc. RNDr. Jan Petr, Ph.D.

Olomouc 2020

ABSTRACT

This doctoral thesis is devoted to development and testing of microfluidic devices from various low-cost materials. The first part of the thesis is dedicated to introduction of microfluidic devices, its history and materials, which are used for their production. Further this part describes production possibilities and detection used in microfluidics. The last part is dedicated to lab-in-a-syringe analysis. The experimental part presents the results of three works. The first part describes the development and testing of laminating film wafer, as an inexpensive alternative to commonly used photolithography, for the production of PDMS devices whose functionality was tested by isotachophoretic separation of two organic dyes - bromophenol blue and amaranth red. The following section describes the development of a low-cost device for multi-parameter chemical analysis of water. This device uses a combination of filter paper with wax printing to create defined channels and reservoirs for individual reactions and a hydrophilic membrane for rapid suction of the sample, the carrier part of the system consists of laminating foils. Selective reagents providing characteristic coloration with the analysed ions are quickly evaluated by the smartphone software application based on HSV (Hue, Saturation, and Value). The application evaluates the amount of analysed ions in the sample (NH_4^+, NO_3^-, NO_2^-, Fe^{2+}, Fe^{3+}, Ca^{2+} and PO_4^{3-}) and pH by comparison with preset calibration values of individual reagents at different analyte concentrations and different sample pH. The last part describes the development of the lab-in-a-syringe test. In this research, we used the experience gained in the manufacture of the previous devices and at the same time enabled detection of the reaction performed directly in the device, which was a major drawback in previously published works. The device consists of a syringe filled with layers of hydrophilic cellulose membrane of various shapes, which serve as a filtration, conjugation and reaction part (detection part). Properties of individual parts of the device were tested on reactions of Ni^{2+} with dimethylglyoxime and Fe^{2+} with 1,10-phenanthroline.

ABSTRAKT

Dizertační práce je zaměřená na vývoj a testování mikrofluidních zařízení z různých nízkonákladových materiálů. První část práce je věnována seznámením se s mikrofluidikou a její historií, materiálům, ze kterých se mikrofluidní zařízení vyrábí. Dále popisuje možnosti výroby těchto zařízení a metody detekce, které se v mikrofluidice používají. Poslední část je věnovaná lab-in-a-syringe („laboratoř ve stříkačce“) analýzám. Následuje experimentální část prezentující výsledky tří prací. První část popisuje vývoj a testování waferu z laminovací fólie, jakožto levnou alternative běžně užívané fotolitgrafie, pro výrobu PDMS zařízení jehož fukčnost byla otestována izotachoforetickou separací dvou organických barviv – bromfenolová modř a amarantová červeň. Následující část popisuje vývoj nízkonákladového zařízení pro multiparametrovou chemickou analýzu vody. Toto zařízení využívá kombinace filtračního papíru s voskovým tiskem pro tvorbu definovaných kanálků a rezervoárů pro jednotlivé reakce a hydrofilní membráni pro rychlé nasávání vzorku, nosnou část systému tvoří laminovací fólie. Selektivní činidla poskytující s analyzovanými ionty charakteristické zbarvení jsou rychle vyhodnocovány pomocí softwarové aplikace chytrého telefonu na základě HSV (Hue - barevný tón, Saturation - sytost barvy a Value - hodnota jasu). Aplikace vyhodnocuje množství analyzovaných iontů ve vzorku (NH_4^+, NO_3^-, NO_2^-, Fe^{2+}, Fe^{3+}, Ca^{2+} a PO_4^{3-}) a pH pomocí porovnání s předem nastavenými kalibračními hodnotami jednotlivých činidel při různých koncentracích analytů a různém pH vzorku. Poslední část popisuje vývoj lab-in-a-syringe testu. V tomto výzkumu jsme využili zkušenosti získané při výrobě předešlých zařízení a zároveň umožnili detekci prováděné reakce přímo v zařízení, což bylo hlavní nevýhodou v dříve publikovaných pracích. Zařízení se skládá z injekční stříkačky naplněné vrstvami hydrofilní celulózové membrány různých tvarů, které slouží jako filtrační, konjugační a reakční část (detekční část). Vlastnosti jednotlivých částí zařízení byly otestovány na reakcích Ni^{2+} s dimethylglyoximem a Fe^{2+} s 1,10-fenantrolinem.

TABLE OF CONTENTS

1. INTRODUCTION

The analytical chemistry is an indispensable part of many industries such as pharmacy, toxicology, food industry, environmental monitoring, and many others. In particular, bioanalytics dealing with the analysis of xenobiotics (substances foreign to the body such as pharmaceuticals, drugs and their metabolites) and biotics (proteins, lipids, nucleic acids, etc.) are often confronted with a limited amount of sample. For this reason, new microfluidic methods have emerged that meet this demand and at the same time are able to deliver robust and reproducible results. First microfluidic chip analysers were developed, these systems are often referred to as the Micro Total Analysis System (μ-TAS). The name μ-TAS expresses an effort to shorten the path of the sample through laboratories and merge as many unit operations as possible into one analyser as big as a credit card. The LOC (lab-on-a-chip) combines all necessary operations such as separation, transport and sample analysis into one chip with channels of several tens or hundreds of micro or nanometers. The microfluidic devices can serve only for pre-treatment of the sample or fully replace conventional analytical instruments providing not only sample analysis but also subsequent detection. We can find their use in practically every area of analytical chemistry.

The dissertation thesis is focused on the development and testing of microfluidic devices from various low-cost materials in connection with colorimetric and smartphone detection. It is based on three original works published in impact factor journals and one patent. One review serves as a basis for the theoretical part. One work is devoted to the development of foil wafer for microfluidic devices made of polymer. The patent describes the development and testing of a portable low-cost device for a multiparameter water analysis. The last work is devoted to the development and testing of the lab-in-a-syringe.

2. THEORETICAL PART

2.1 WHAT ARE MICROFLUIDIC DEVICES?

Microfluidic devices are basically technological systems that manipulate small (10^{-9} to 10^{-18} litres) amounts of fluids into channels with dimensions of tens to hundreds of micrometres.[1] Microfluidic chip is a set of micro-channels etched or molded into a material (glass, silicon, polymer etc.), connected together in order to achieve the desired features for fully automated analytical system, such a devices are also known as Micro Total Analysis System (μ-TAS). Comparison between size of a micro- or a nanofluidic device with other common entities is shown on Fig. 1

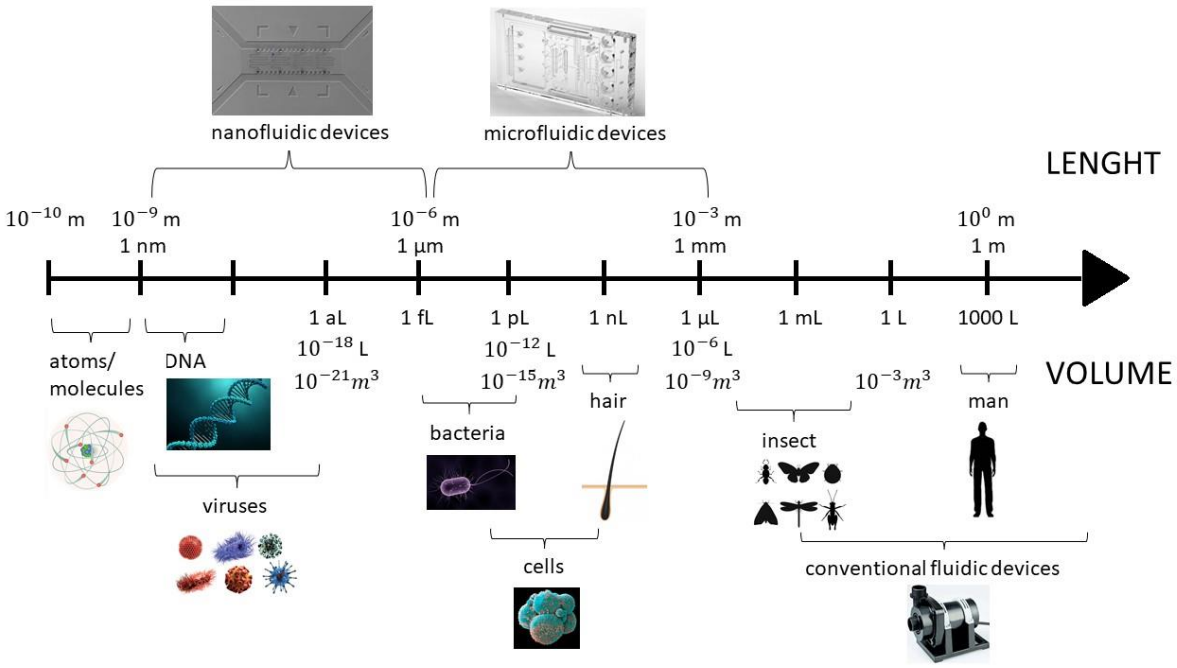

Figure 1. Comparison between size of a micro- and a nanofluidic devices with other common entities

This technology started with trend of miniaturization of analytical methods within approx. last 30 years. First of all, the analysers were downgraded to apparatus, which were able to be placed on working table. Subsequently the hand (portable) analysers have appeared. In recent years, we have been going over to analysis on chip, so called Lab-on-a-chip (LOC). Micro and nanochips are final stadium of this process nowadays. Miniaturization has numerous

advantages: e.g. reduction of acquisition and operating costs (energy consumption, reagent volume, etc.) of individual devices, easy handling and controlling, spatial ease in the lab and beyond. All these aspects lead to cheaper price of the lab analysis. We still have to consider all the processes so that it will not lead to a reduction of the quality of the obtained results. Therefore, all microfluidic devices are developed to be able to compete with instrumental methods by their detection limits and other analytical characteristic. [2].

2.2 WHAT WERE THE FOUNDATION STONES OF MICROFLUIDICS?

In 1949, Müller and Clegg patterned a filter paper with a paraffin barrier and observed speeding up of the diffusion process of the sample caused by the confined channel and reduced sample consumption, therefore this research was regarded as one of the basis of paper microfluidics. [3] In 1956, the first paper device for the semi-quantitative detection of glucose in urine was presented by J. P. Comer.[4] On the other hand the first microfluidic application in industrial environment occurred in 1980s and were triggered by the generalization of ink jet printing and micro electromechanical systems (MEMS) used for example in pressure sensors. In 1979, Hewlett Packard developed the Thermal Inkjet Technology and MEMS technology used to manufacture the nozzles. [5,6] The microfluidic appeared during last hundred years in various disciplines, but based on professor Whitesides theory, it originated in following four fields: molecular analysis, biodefence, molecular biology and microelectronics. First were analysis, as mentioned above, there was trend of miniaturization in all analytical methods like GC, HPLC and CE, which in capillary format revolutionized chemical analysis. The second motivation came from the field of molecular biology. In this discipline the need of analytical methods with much greater throughput and higher sensitivity and resolution for DNA sequencing and other genomics emerged in 1980s. Best solutions to overcome these problems were offered by microfluidics. Third motivation was danger of chemical and biological weapons after the cold war. In 1990s, Defense Advanced Research Projects Agency (DARPA) of the US Department of Defence supported many programmes aimed at developing portable microfluidic systems for detection of chemical and biological threats. The last contribution was from microelectronic, where photolitography and associated technologies were successful in silicon microelectronics and MEMS. The original motivation was to directly apply them into

microfluidics. In 1990 also Manz et al. presented Miniaturized Total Analysis System (μTAS), which periodically transforms chemical information into electronic information. Sampling, sample transport, chemical reactions, chromatographic separation and detection are automatically carried out in such a device. The detector or sensor in a TAS does not need high sensitivity, because sample pre-treatment serves as elimination of most of the interfering chemical compounds and impurities. Calibration can be incorporated in the system as well.[7] Some of the earliest works used silicon and glass in fluidic microsystems, but later polymers became the most widely used materials for microfluidics fabrication. [8–11]

2.3 WHAT IS LAB-IN-A-SYRINGE?

The lab-in-a-syringe (LIS) technology represents an innovative methodology in the field of biosensing based on the concept of lateral- or vertical-flow assays and microfluidic paper-based devices (μPADs). The concept of lateral flow assay (LFA) tests are well-known from the common pregnancy tests, aimed at detection of the presence of hCG (Human chorionic gonadotropin). In vertical-flow assay (VFA) tests, is the flow of liquid vertical compared to the LFA. They are formed by the layers stacked on the top of each other, similarly as layered or "origami" μPADs.[12,13] The LIS concept can only serve for sample pre-treatment such as extraction, filtration before conventional analytical analysis or involve the entire analysis inside the syringe. The main problem of the LIS concept lays in the detection. During the LFA tests is the detection pad easily visible for users. But in LIS tests, user must open the cartridge, holding the detection pad. This represents the crucial practical problem in case of analysing potentially harmful or infectious samples.

3. AIMS OF THE THESIS

The topic of the doctoral thesis is the study, production and testing of microfluidic devices from various low-cost materials. Particular task included:

- extend the knowledge from MSc research by creating the low-cost test for water analysis from laminating foil and paper,
- extend the knowledge from Ph.D. internship, which included developing the solution of PDMS wafer fabrication from low-cost material,
- apply the acquired knowledge of the production of microfluidic devices in combination with other materials, including the use of syringe,
- testing of fabricated devices on analysis, which could be used in Third World countries,
- studying of application of smartphones in connection with detection.

These goals led to publication output:

- **Lenka Hárendarčíková**, Daniel Baron, Andrea Šebestová, Jan Rozsypal, Jan Petr, True lab-in-a-syringe technology for bioassays. Talanta 174 (2017) 285-288.
- **Lenka Hárendarčíková**, Jan Petr, Fabrication of low-cost polydimethylsiloxane master from laminating foil for isotachophoresis separation on a chip, Instrumentation Science & Technology, 46 (2018) 316-325.
- **Lenka Hárendarčíková**, Jan Petr, Smartphones & microfluidics: Marriage for the future. Electrophoresis 39 (2018) 1319-1328.
- Palacký University Olomouc. A portable low-cost device for a multiparameter chemical analysis. Originators: Jan Petr, Vítězslav Maier, Martin Švidrnoch, **Lenka Hárendarčíková**, Czech utility model 30744, registered at Czech Industrial Property Office Prague on 13th June 2017.
- Palacký University Olomouc. A portable low-cost device for a multiparameter chemical analysis. Originators: Jan Petr, Vítězslav Maier, Martin Švidrnoch, **Lenka Hárendarčíková**, Czech Patent 307 248, registered at Czech Industrial Property Office Prague on 14th of March 2018.

4. EXPERIMENTAL PART

4.1 FABRICATION OF LOW-COST POLYDIMETHYLSILOXANE MASTER

Most of the data presented in this section have been also published in ref. [14]

Chemicals

3-Ton Clear epoxy adhesive was purchased from Alteco (Broumov, Czech Republic). Methanol was purchased from Sigma-Aldrich (Prague, Czech Republic). Polydimethylsiloxane and curing agent (Sylgard 184) were purchased from ELCHEMCo (Zruč nad Sázavou, Czech Republic). Bromphenol Blue, amaranth red, hydrochlorid acid, 2-(N-morpholino)ethanesulfonic acid and glycine were of p.a. quality and purchased from Sigma-Aldrich (Steinheinm, Germany). Deionized water of 18.2 MΩ cm was produced from MilliQ (Millipore USA).

Master fabrication

The Fellowes office laminator Mars A3 and laminating pouches Fellowes (Itasca, IL, USA) were laminated together. After the bonding, carved for Petri dish size (the final device was casted inside). Carved foils were washed with distilled water, methanol and dried using compressed air. Clean wafer base – foil circles - were cut in desired structure with fabricated knife fixed in the desktop corner cutter (Warrior, Taichung. This knife was made from two technical trapezoidal knives with triangle shaped blades, which were precisely glued together by epoxy adhesive. The glued „double-knife“ was cured overnight. Scheme of the fabricated knife with size data is shown in Fig. 2.

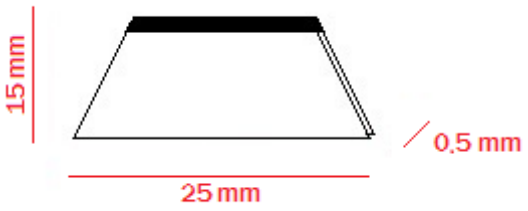

Figure 2. Scheme of fabricated knife with dimensions

The fixation of the cutter ensures the use of the same cutting pressure and better reproducibility of the depth of the channels (the carved structures could not penetrate the whole foil depth). After the cutting process, the cleaning procedure was repeated. In the final product, the microfluidic channels arise between those cuts. The dimensions of the wafer were analysed using the contact profiler Form Talysurf Series 2 (Leicester, UK).

Curing of PDMS and device preparation

Prepolymer of PDMS and curing agent were mixed in the ratio of 10:1, stirred properly and then degassed in vacuum. [15] The time of degassing depends on the size of the wafer. The process is finished, when no bubbles are visible on the surface of polydimexyl siloxane layer. Petri dish was covered with aluminium foil to protect the glass from prepolymer mixture. Next, the foil master was fixed into Petri dish before pouring of PDMS because of density and weight of the foil, it did not stay at the bottom, but floated on the prepolymer layer. Fixture was done by spreading a layer of prepolymer mixture on the aluminium foil (or master from the bottom) and cured in the oven at 70°C for 10-20min. The layer of PDMS held the master and afterwards, the prepolymer was poured onto the master and cured at 60°C over night.

The cured PDMS slap with defined structures was peeled off from the master, the reservoirs were punched with biopsy punch 1.5 mm size (from Miltex, USA) and cut for single device. The cut devices and microscope slides were washed with methanol and dried with compressed air. Manipulation with both parts was performed with tweezer to protect it from dust and other impurities. Final device was sealed with air plasma Zepto Rie (from Diener Electronic, Germany), which stuck the microscope slide and PDMS layer together. The pressure of the air used in plasma cleaner for bonding was fifty mbar for thirty seconds. Whole process is drawn in Fig. 3.

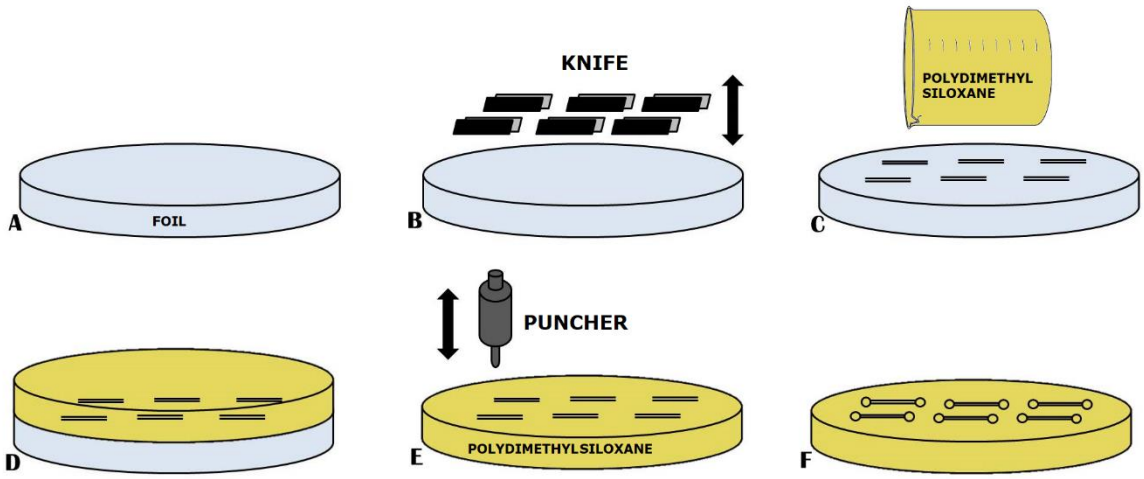

Figure 3. Scheme of the wafer and PDMS chip fabrication: (A) carved laminating foil, (B) punching of channels with customs made knife, (C) punched foil and prepolymer pouring, (D) PDMS curing, (E) reservoirs punching and (F) punched PDMS before cutting for single devices.

Isotachophoresis (ITP)

Isotachophoretic separation of two organic dyes – bromphenol blue and amaranth red was studied to prove the concept of low-cost master fabrication. In isotachophoresis, the sample is injected between two electrolytes. One electrolyte contained the fastest co-ion with the highest mobility (leading electrolyte) and second contained the slowest co-ion with the lowest mobility (terminating electrolyte). After application of an electric potential charged analytes are separated based on ionic mobility (this factor tells how fast an ion migrates through an electric field), during separation process the analytes create zones and in the final steady state these zones are arranged in order of their mobility and migrate with same velocity. Migration time is dependent on the mobility, capillary length and electric current. Sharp boundaries between neighbouring zones are typical for ITP as a result of jumps in voltage between them. This principle shows Fig. 17 in part „results and discussion“. [16]

Sample preparation

The solution of 10 mmol L^{-1} hydrochloric acid served as the leading electrolyte and 10 mmol L^{-1} 2-(N-morpholino)ethanesulfonic acid served as terminating electrolyte. The pH of both electrolytes was modified to 4.8 by glycine. The sample composed of mixture of 100 µL of bromphenol blue (10^{-3} mol L^{-1} in deionized water), 100 µL of amaranth red (10^{-3} mol L^{-1} in deionized water) and 100 µL of the leading electrolyte.

Results and discussion

Wafer fabrication

After previous combination of lamination foil and paper or membrane, there was idea of using laminating foil for production of the whole microfluidic device, by stacking layer of foil between two, which were sealing them into final device. Advantage of this principle was that part inside, which were creating shape of the channels, could be cut in different shapes, from thicker or thinner foil. Overall, we can prepare a diverse range of microfluidic channels. But the main disadvantage is that foils are glued (by thermobonding in laminating machine), so for e.g. if you try to fill the foil with hot agarose to prepare channels for gel electrophoresis the structure of the channel will be destroyed. Figure 4 shows early studies of the behavior of laminating foil devices on a larger scale. All devices were filled with agarose (1% m/v) colored with brilliant blue dye (Merck, Czech Republic) for better visualization of leakage after hot filling.

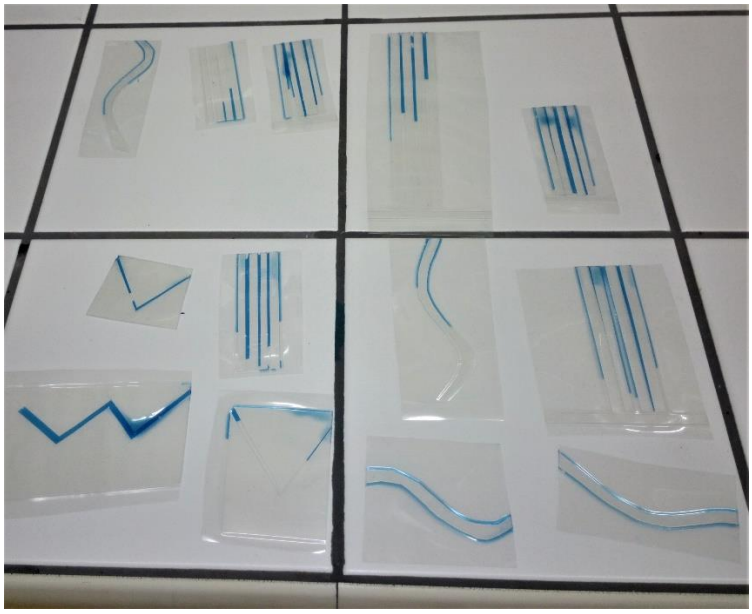

Figure 4. Different shape of channels in foil filled with colored agarose gel

After several similar results from other experiments, the decision was made, that it would be better to use lamination foil only as a base for PDMS device. Lamination foils were chosen as a base material for masters preparation because of their inertness, smoothness (which is necessary for the foil to be non-stick with cured polydimethylsiloxane) and flexibility. We have studied five foils widths: 80, 100, 125, 175 and 250 µm. The grooves were cut onto all foils and then the PDMS chip was fabricated, as described above. Afterwards the grooves were analysed under the microscope and it was found that the best repeatability of the groove structure was obtained using 100 µm foil width. This is probably due to the strength of the laminated foil and the pressure needed for the channel preparation.

The next parameter tested was the use of the fixed knives in the desktop corner cutter or the use of fine precise hand cutting. The hand cutting was very repeatable, when one person repeated the procedure, the relative standard deviation of the channel width was about 5%, n=10. But the reproduction capabilities rapidly dropped, when two people repeated the procedure. The relative standard deviation of the channel width was over 80%, each n=10. The foils are tough enough to hold the desired shape but also not tough enough to be punched to desired structure. Although, it seemed that the desktop corner cutter is not fit for fixing the knives. However, we found that the key step in the procedure is cutting, hence, the knives were fixed in the desktop corner cutter under the small angle to ensure the cutting the foil, not

punching. This led to the reproducible formation of grooves with the similar precision as was for one person hand cutting.

The knives, as described before, form two straight grooves with triangle cross-section (typical for cutting) having width of 90 ± 8 µm and depth of 51 ± 6 µm. The distance between them is 344 ± 4 µm as shown Figure 5. All values are calculated from 5 different grooves, each measured in three positions. Average value with standard deviation is displayed.

Within the fabrication of PDMS chips, the cut grooves (negative depth from the base) formed PDMS images (positive depth from the base; „hills“), which were then bonded on the glass slide forming a channel between both „hills“. The channel width was still quite precise, 326 ± 41 µm. Although, the channel precision and resolution cannot compete with traditional lithographic procedures but it can allow analytical procedures where precision is not the key parameter and it is significantly cheaper, e.g., using such devices as sample pre-treatment tools or for specific separations; e.g., where the channels are filled with agarose or polyacrylamide gels. [17]

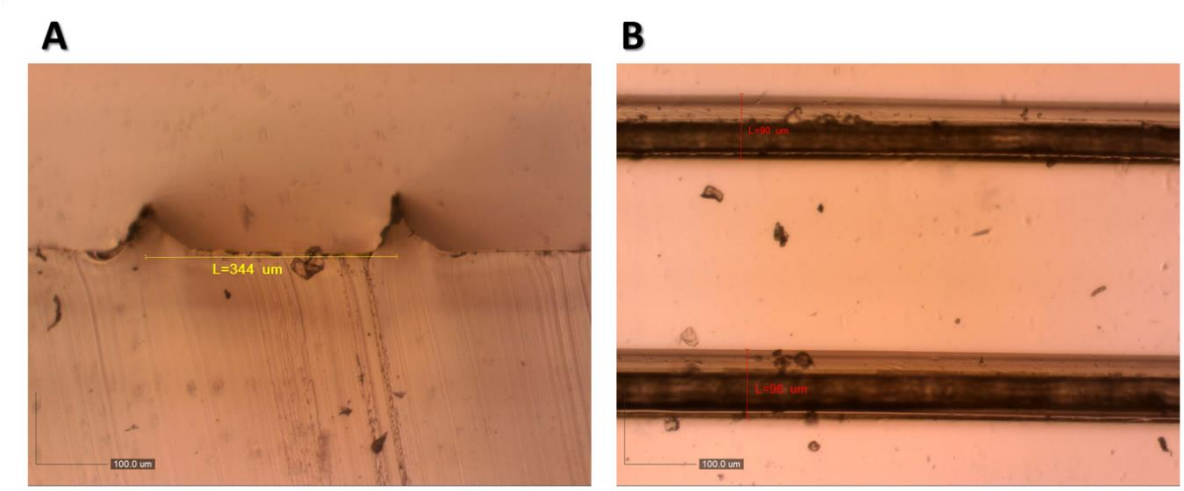

Figure 5. Fabricated channels: (A) cross-section of the cut channels, (B) top-view of the cut channels

The width of the channel can be easily varied by changing the space between the glued knives, for example by putting an extra slice of metal between them or by using smaller or thinner knives. Picture of the whole device with smaller channels is shown in Fig. 6.

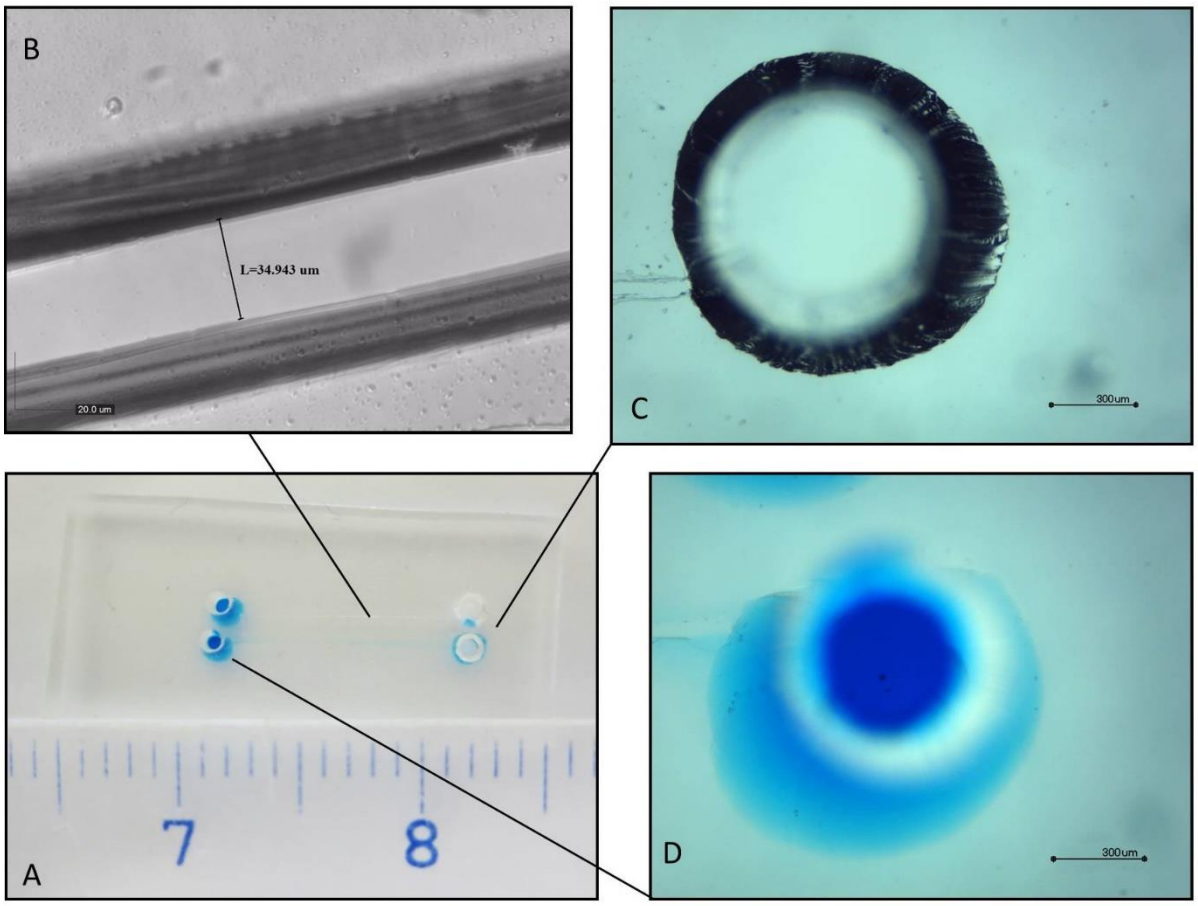

Figure 6. (A) Complete device (photo took with Canon PowerShot S110, **(B)** magnified channel (FLIM, fluorescence microscope, IntracoMicro), **(C and D)** magnified image of empty reservoir and filled one with brilliant blue dye (Microscope VSM 52, IntracoMicro)

3D view of channel with different dimensions of used knife, we can see thanks to image from 3D microscope VHX-6000 (Keyence, Japan) on Fig. 7.

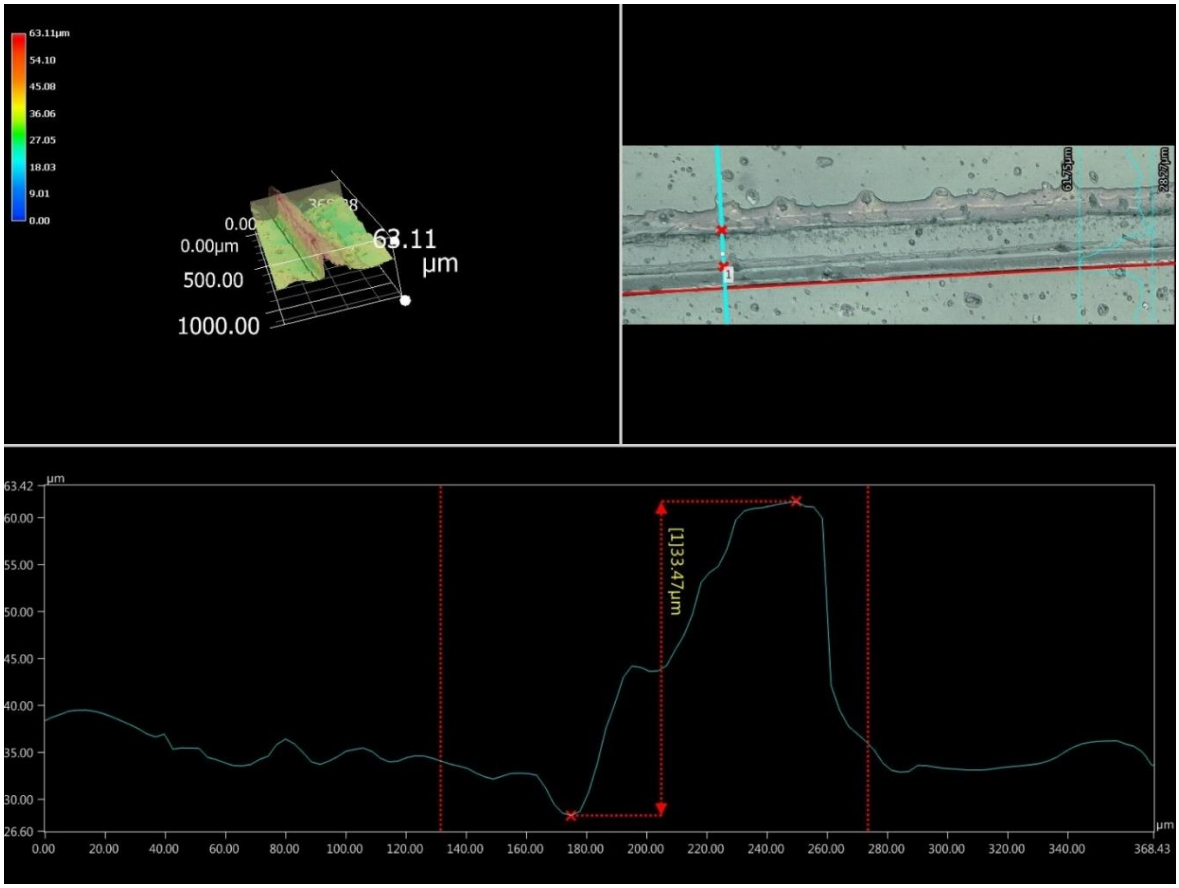

Figure 7. 3D view of microfluidic channel fabricated with our solution

Isotachophoretic separation

To prove the concept of our device we chose model separation of two organic dyes. Isotachophoresis system was established according to the previously published conditions. [18,19] The analysis was running in the straight channel device with the channel length of 2.5 cm with 10 mmol L^{-1} HCl as the leading electrolyte and 10 mmol L^{-1} 2-(N-morpholino)ethanesulfonic acid as terminating electrolyte, both at pH 4.8. The whole device was filled with leading electrolyte after polydimethylsiloxane bonding to avoid the loss of channel hydrophilicity. The subsequent isotachophoretic separation process is described step by step in Figure 8. At first, the leading electrolyte was replaced with the sample solution and the electric field of 20 V cm^{-1} was applied for 45 s. After sample loading, the reservoir was emptied and rest of the sample solution was washed out by the terminating electrolyte. Afterwards, the reservoir was filled with terminating electrolyte and the electric field

of 20 V cm^{-1} was applied again to start the separation process. After a few seconds, the sample movement was apparent, around a minute after voltage applying, the separation process began and after approx. 3 min, the separation of two organic anionic dyes was achieved as shown Figure 9.

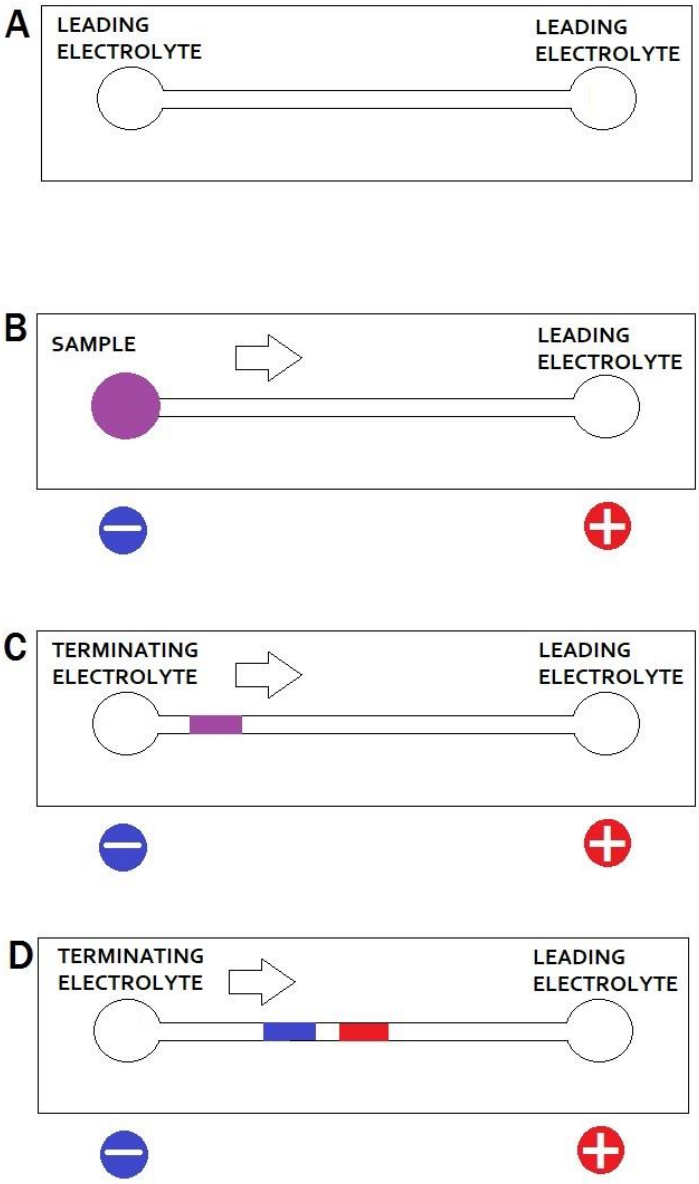

Figure 8. Scheme of isotachophoretic process: (a) Device filled with leading electrolyte, (b) reservoir filled with sample and sample loading, (c) reservoir filled with terminating electrolyte, and (d) separation

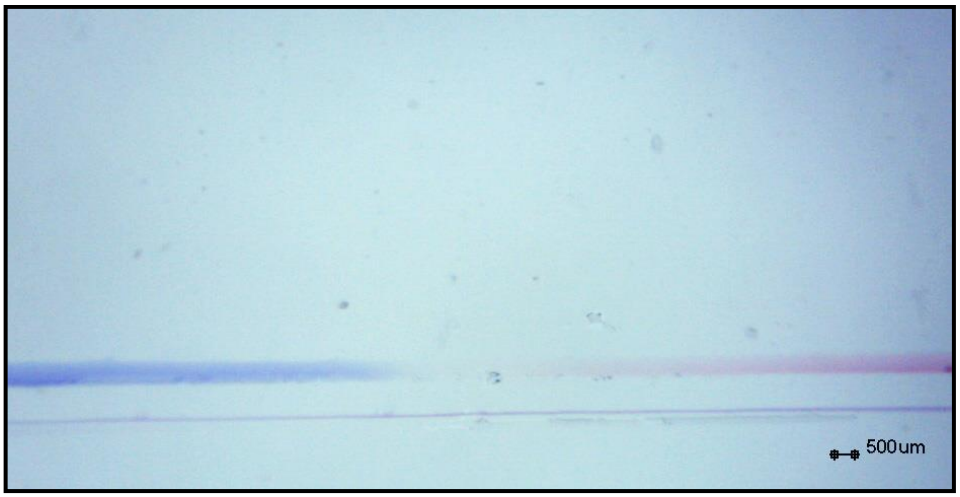

Figure 9. Example of isotachophoretic separation of bromphenol blue and amaranth red.

Conclusion

The new master fabrication process allows us to produce foil master with channel dimensions of dozens of microns. The main advantage is the possibility to quickly modify the design and shape to perform the desired analysis even in low-cost laboratories or studies, where a cleanroom and expensive instruments are not available. The preparation time is also incomparable, because preparation of the master (excluding knife fabrication) takes just couple of minutes, and besides cleaning there is no additional step necessary. The net costs of wafer preparation were approx. 200USD, which cover office laminator, box of laminating pouches (100 pieces) and customs made knife. At this price, we could prepare 600 masters because from one foil we were able to prepare six masters of Petri dish size. It means, the costs of one wafer was around 0.34 USD. There are also advantages such as fabrication time and simple channel design changes. The advantages of our wafer, compared with the one fabricated with photolithography are summarized in Table 1, all prices are approximate.

Table 1: Summarization of photolithography and foil wafer processes

Parameter	Photolithographic wafer	Foil wafer
price of one wafer	**32 USD**	**0.34 USD**
chromium mask for photolithography	**319 USD**	**0 USD**
time of fabrication / amount of fabricated wafers	**120 min / 5**	**15 min / 6**
operating costs of fabricating (2 hours)	**85 USD**	**0.23 USD**
possibility to change the mask?		**YES**
need of cleanroom	**YES**	**NO**

4.2 PORTABLE LOW-COST DEVICE FOR MULTI-PARAMETER CHEMICAL ANALYSIS – AQUACHIP

Most of the data presented in this section have been also published in ref. [20,21]

Chemicals

Reagents for pH 3-10 and 9-13 and sulfuric acid were purchased from Fluka (Prague, Czech Republic). Nessler´s reagent, sulfanilamide, hydrochloric acid, N-(1-naphthyl)ethylenediamine dihydrochloride, chromotropic acid disodium salt dihydrate, calconcarboxylic acid, 1,10-phenantroline monohydrate, ammonium thiocyanate, hydrazine hydrate, sodium chloride, sodium fluoride, sodium nitride, silver nitrate, ammonium hydroxide solution, calcium chloride, Iron(III) chloride hexahydrate, Iron(II) chloride tetrahydrate, sodium sulfate anhydrous, potassium iodide, natrium hydroxide, phosphoric acid, zirconium(IV) chloride and poly(ethylene glycol) 3000 were purchased from Sigma-Aldrich (Prague, Czech Republic). Methanol was purchased from VWR Prolabo chemicals (Rožnov pod Radhoštěm, Czech Republic). Schwarzenbach buffer was purchased from Mikrochem Trade (Pezinok, Slovakia). Ammonium molybdenane tetrahydrate was purchased from Lach-Ner (Neratovice, Czech Republic). Deionized water of 18.2 MΩ cm was produced from MilliQ (Millipore USA).

Sample preparation

Basic ion solution was prepared in volumetric flasks of 10mL volume by dissolving solid salts or diluting the concentrated solution to concentration 2 mol L^{-1}. This basic solution was used for preparation of a set of calibration solutions. Calibration was done in range from 0.25 mol L^{-1} to 2 mol L^{-1}.

The pH reagents and Nessler´s reagent have been prepared by the manufacturer and used directly for testing. For determination of nitrites mixture of solutions in ratio of 1:1 was used. For the first solution 0.5g sulfanilamide and 5 ml of HCl (32%) were dissolved in deionized water and made up to 50 ml in a volumetric flask. Second solution was 0,05 g of N-(1-naphthyl)ethylenediamine dihydrochloride dissolved in deionized water and made up to 50 ml in a volumetric flask. The nitrates reagent consists of 1 g of chromotropic acid disodium salt dihydrate dissolved in deionized water (50 ml volumetric flask).

For determination of calcium ions was used mixture in ration of 1:1. One part of this mixture was 250 mg of calconcarboxylic acid diluted in 50ml of methanol and second part was Schwarzenbach buffer. 0,2 % solution of 1,10-phenantroline monohydrate in 0,1 mol L^{-1} HCl was used for ferrous ions. For ferric ions 0,1 mol L^{-1} solution of ammonium thiocyanate in deionized water was used. [22–25]

Aquachip fabrication and use

Aquachip consists of three basic parts – carrier part (see Fig. 10-1), reaction part (Figure 10-8) and a part, which ensures the transport of the sample to the reaction zones (see Fig. 10-16). On a filter paper (Whatman Grade 1) used as a base for reaction cells is printed the structure of reaction part. Wax printer (Xerox ColorQube) is used to print the hydrophobic pattern (see Fig. 10-8) onto filter paper. Since t

he printed wax layer is only on the surface and not through the entire paper structure, it is necessary to press the paper and place it in the oven or iron it between a non-sticky layer such as baking paper. After the formation of wax barriers, the paper is cut into three reaction parts (see Fig. 10-9, 10-10 and 10-11).

Carrier part consists of lower covering layer (see Fig. 10-2) which is formed from credit card size laminating foil, in which instructions for working with the device can be laminated from the back side of the device (we were not using it in our experiments). Furthermore, only a half of the laminating pouche with the adhesive layer (see Fig. 10-3) is used to create upper cover piece (see Fig. 10-4), having a cut shape corresponding to other parts of the device. Under this layer name of device and instructions of maximum line for immersion of the device in the analysed sample can be placed (see Fig. 10-26). The notch on Figure 10-6 corresponding to the insertion of the reaction portion and the notch (see Fig. 10-7) corresponding to insertion of the sample transport pads (see Fig. 10-17 and 10-18) to the reaction zones.

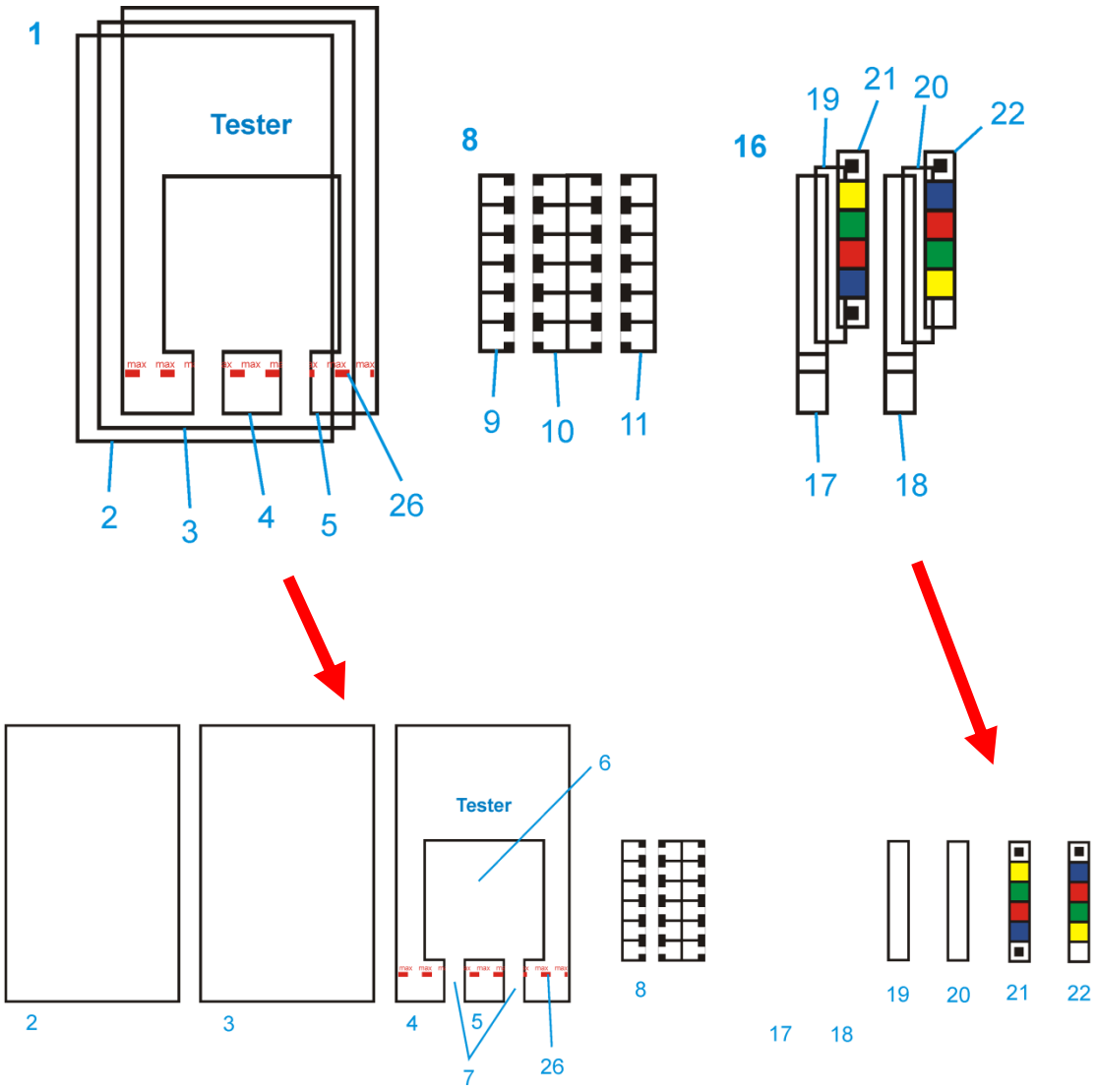

Figure 10. Individual parts of Aquachip

Figure 11 shows further details of structure of the reaction portion. Reaction portion is formed from square reaction zones (see Fig. 11-12) having a thin edge (see Fig. 11-13) on three sides and a wide edge (see Fig. 11-14) on one side with a gap (see Fig. 11-15). To perform the analysis, specific reagent for a particular test parameter is applied to the square space, to fill the entire square space. The hydrophobic wax barriers ensure no liquid transfer between the reaction zones. Also, the amount of liquid applied must not exceed the content of the square, because then it would contaminate other parts of the device through the gaps. Reagents are applied according to their characteristics, some need to be dried immediately, some may partially break the barrier and therefore it is not possible to apply them to the entire area of the square. The liquid sample to be analysed flows through the gap to the reaction site

(square with reagent). The reaction part is aligned and glued onto the cut-out on the upper cover part of the support part of the device.

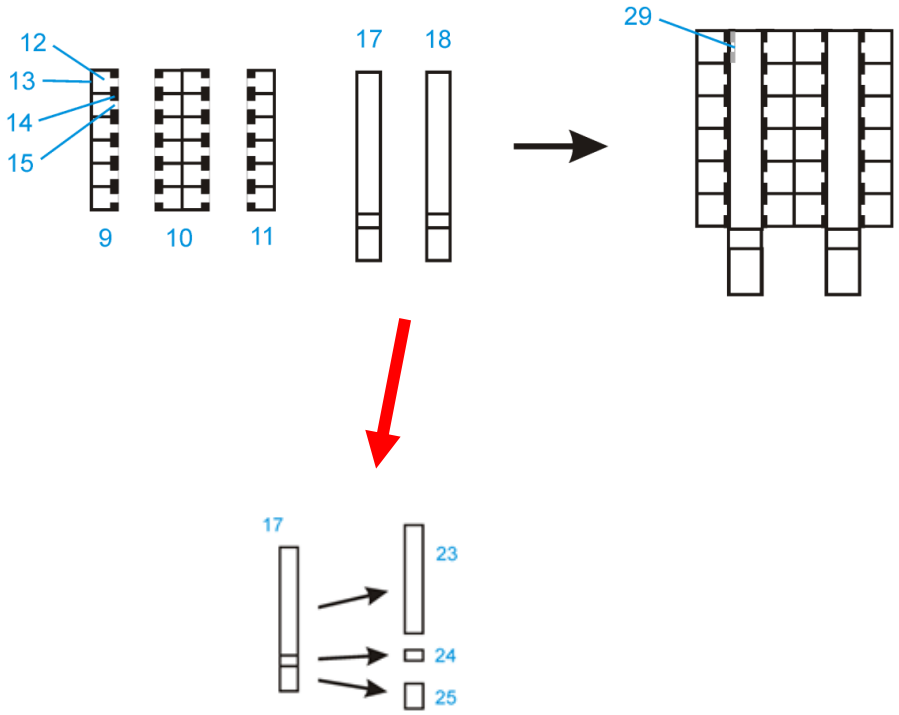

Figure 11. Detail of the reaction zone and transport pads

The „sample transport part“ is composed of three aligned parts which are on the apparatus in two identical copies, because the samples are transported simultaneously by two routes. Specifically, there are following parts: the hydrophilic layers leading the sample to the reaction zones and dispensing the sample (see Fig. 10-17 and 10-18), adhesive layers (see Fig. 10-19 and 10-20) and evaluation layers (see Fig. 10-21 and 10-22). A hydrophilic layer is a cellulose fiber membrane (CFSP203000 from Millipore, Labicom Czech Republic) used to absorb samples in test strips. This layer may be either non-divided or divided into multiple portions. Figure 11-23 serves as a transport of the sample to individual reaction zones. The middle portion Figure 11-24 modifies the sample and removes the impurities from the sample. It can be impregnated with agents causing such changes. Its location is below the beginning of the reaction portion of the device. The same can be performed in other parts of the device. However, in the case of chemical modification of the sample, including for e.g. pH adjustment, it must be carried out at a point where no other contamination occurs in the reaction zones. The lower part (see Fig. 11-25) is used to suck the sample. Its size,

respectively length is determined by the maximum immersion of the device in the sample. If the intermediate portion would be below the maximum immersion limit of the device, there is a risk that the sample will not be completely modified by the reagents contained in the intermediate portion. The hydrophilic layer is flushed and glued into the cut-out (see Fig. 10-7) on the upper cover part of the support part of the device. It is essential to align this layer with the reaction part of the device on the reaction part, where it covers part, mostly half of the width of the wider side of the reaction zones. This will ideally overlap the portion that transports the sample to the reaction zones and entre the reaction zones. If such overlapping did not occur, the analysis would not be fast enough and there would be risk of contamination of the reaction zones with other reagents from the other reaction zones.

The evaluation zones (see Fig. 10-21 and 10-22) consist of paper with printed squares of the same dimensions as the squares of reaction zones. Three outer squares (see Fig. 12-27) define the position of individual reaction zones on the whole tester for evaluation by smartphones or tablets. The middle squares (see Fig. 12-28) are then used to set the color range for the evaluation software. The length of evaluation layer may be the same as the length of reaction portion or in case of divided hydrophilic sample dispensing layer may be extended to the point of maximum immersion of the device, so that the separation of the layer will not perceptible to the user. But evaluation layer can not be longer than maximum immersion site, because the printed text or colors would be washed away and would contaminate the sample.

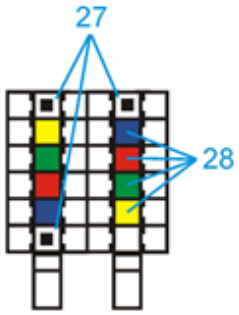

Figure 12. Detail of evaluation zones

Software

The WAXAN evaluation software (for Android platform) is capable of following actions: automatically detects the reaction field of the chip, marks it and cuts the reaction field to avoid the detection of a „black border“, that would make analysis difficult. Then it evaluates the color of the captured pixels, converts them to HSV format (hue, saturation, value), and compares them to the predefined range directly in the software and displays the value of the parameter (e.g. concentration of analyte).

The software does not calibrate the ambient light intensity, it automatically triggers illumination through the telephone before capturing the image (photo). This creates relatively the same illumination conditions.

Results and discussion

At the early stages, before the Aquachip design we have started by choosing the right reagents for our ion analytes. The idea of smartphone detection was already in our mind, so we were focusing on reactions with significant color change, which will be easy for detection. At first, we were trying our reaction with above mentioned reagents on a drip plate (see Fig. 14A and 14B). Only difference here is in determination of nitrates. The first was used zirconium chloride solution. The product of such reaction is a white precipitate.

The white precipitate is possible to use for example on black filtration paper, but it is impossible to judge the concentration of analyte because the color intensity is not changing with different concentration of analyte and also the color of filtration paper is not stable through whole surface. Reaction on filtration paper shows Fig. 13.

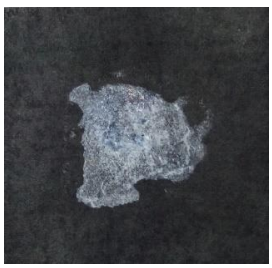

Figure 13. Reaction of zirconyl(IV) chloride with nitrates (concentration 2 mol L^{-1}).

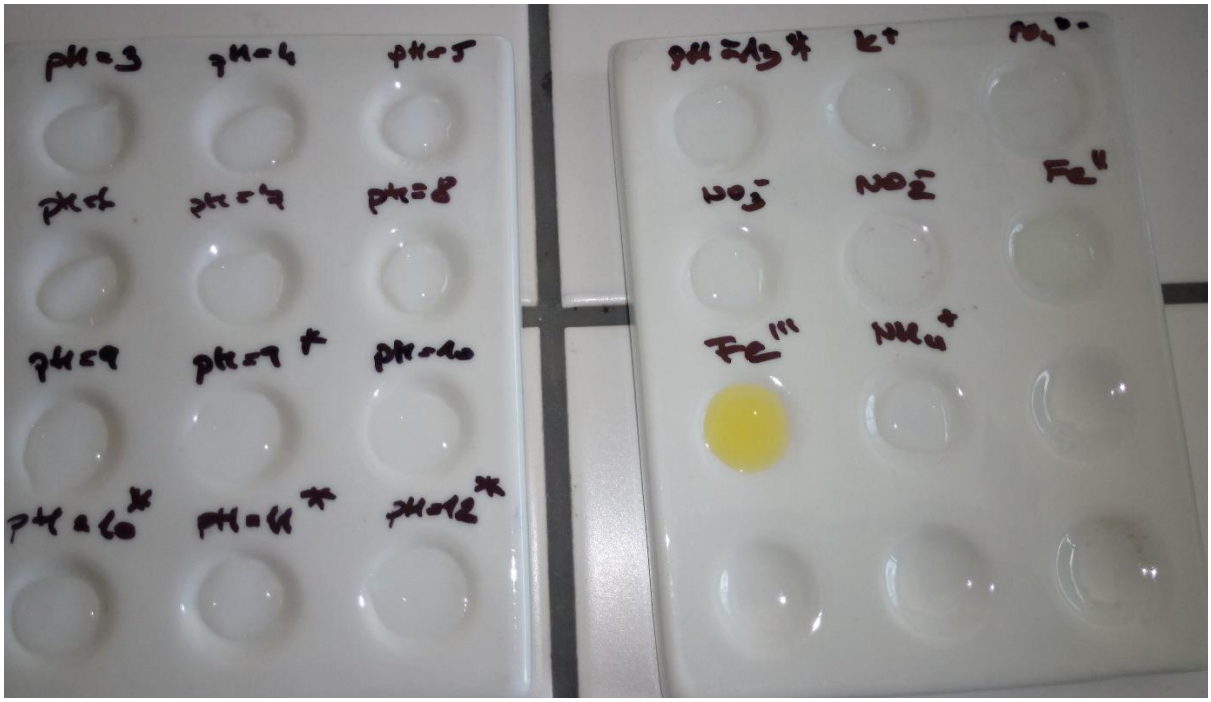

Figure 14A. Drip plate with ion and pH samples before reaction

Figure 14B. Drip plate with ion and pH samples after reaction with appropriate reagents (pH with star mark means use of pH reagent with range 9-13)

Following step was to select the appropriate material and chip arrangement. The material serving as the substrate for the reaction is the most essential part of the whole apparatus. There must be no changes, especially on the surface, due to reactions. The first we considered thicker materials to be able to use a larger sample volume for the reaction itself. First, we tested the plate for liquid chromatography (aluminium foil with anchored stationary phase/slurry). This solution has two major problems; first we are not able to stop or control liquid flow only in „reaction zones“. We tried to scratch the grid, but then the stationary phase became crumbled. Other issue is reaction of ammonium ions with Nessler´s reagent, which destroy stationary phase. Both problems are shown in Figure 15.

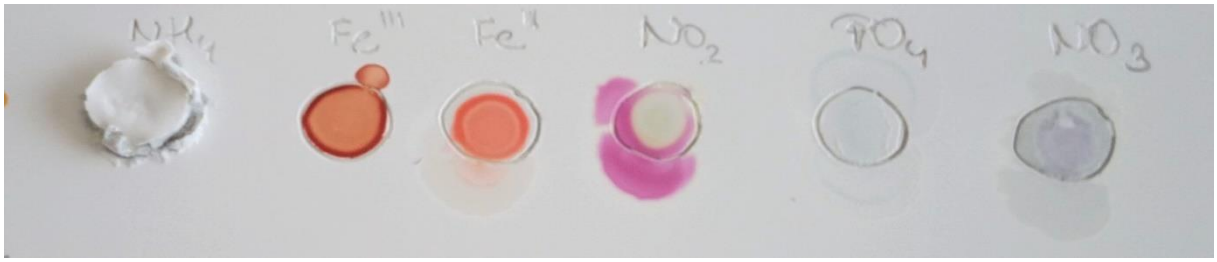

Figure 15. The leakage of reactions and releasing of stationary phase in reaction of Nessler´s reagent with ammonium ions

Because we wanted to support the speed of sample flow, another tested material was cellulose fiber membrane (CFSP203000 from Millipore). Significant advantage is the sample flow rate, but the reaction itself may not be visible on the membrane surface due to its thickness (for example see Fig. 16 shows like 3 different pH values are not possible to recognize in comparison with colors on drip plate), which is necessary for subsequent evaluation by the software on the smartphone. Unlike conventional filter paper, which were eventually used for the reaction zone, we are not able to create a wax barrier through the entire membrane layer, resulting in the reaction zones suffering from leakage. At the end we decided to use filtration paper and wax barrier, as already described above. We have tested different sizes and shapes of the channels in whole device, the division of the reaction zones similar to „QR code“ is ideal to be processed by the software.

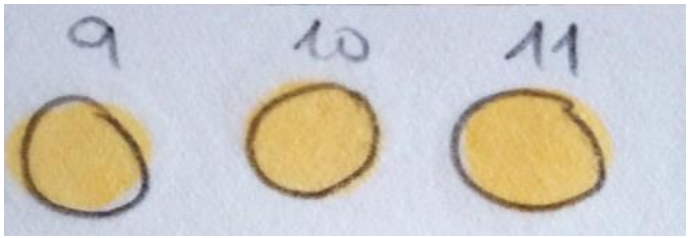

Figure 16. pH 9-11 reaction on cellulose fiber membrane

We also wanted to use the knowledge gained from previous research concerning the implementation of laminating foil, which was meant to isolate the whole analysis from the environment. But when the whole device was sealed, it suffered from leakage and reaction mixing around cellulose fiber membrane which served for sample transport to the reaction zones. In addition, we did not want the reaction zone to pass through the laminator due to possible thermal degradation of the reagent, thus laminating foil was used as carrier part.

Within the prototype of a portable low-cost device for multi-parameter chemical analysis, individual positions were occupied by reaction tests for the analysis of pH, ammonium, calcium, nitrate, nitrite, phosphate and ferrous and ferrous ions. Positions are listed in Table 2 and reaction part division shows Figure 17.

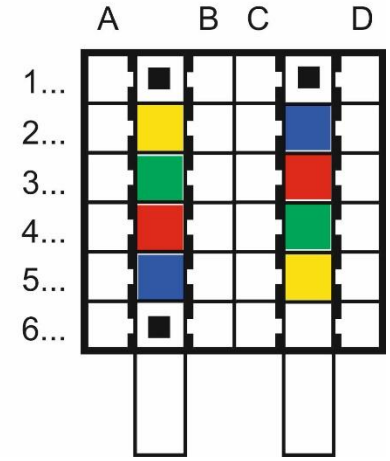

Figure 17. The division of reaction part

Table 2: Position of tests on the chip

Position	Test
1A	pH 3 – 10
1B	NH_4^+
1D	pH 9 – 13
3A	NO_2^-
3C	Ca^{2+}
3D	NO_3^-
5A	Fe^{2+}
5B	PO_4^{3-}
5D	Fe^{3+}

For test preparation 0,5 µL of reagent is always applied per reaction site. In case of mixed reagents, 0,5 µL of mixture is applied to the reaction site (all reagents are mentioned above, together with preparation processes). Afterwards the reaction part is dried under a stream of hot air using a hair dryer. Final device is then immersed in water sample containing analytes and after reaction, picture is captured via smartphone camera and reaction and analytes concentration is evaluated. How the real device looks like shows Figure 18.

Figure 18. Real AquaChip photos from both sides

The evaluation of concentration is based on comparison with calibration solution of each ion consisting of concentration range from 0.25 mol L^{-1} to 2 mol L^{-1} and the result color

of their reaction with reagents in reaction zones, also the color of background was captured for the same purpose. Analytical parameters of AquaChip shows Table 3.

Table 3: Analytical parameters of AquaChip

Analyte	Concentration range	Accuracy*
pH	3 – 13	Δ pH 3
NH_4^+	130 – 1200 mg L^{-1}	30 mg/L
NO_2^-	140 – 920 mg L^{-1}	30 mg/L
NO_3^-	190 – 930 mg L^{-1}	40 mg/L
Ca^{2+}	200 – 800 mg L^{-1}	40 mg/L
Fe^{2+}	170 – 2790 mg L^{-1}	35 mg/L
Fe^{3+}	140 – 1400 mg L^{-1}	30 mg/L
PO_4^{3-}	240 – 2850 mg L^{-1}	50 mg/L

* Accuracy depends on software calibration, generally in the order of 20 - 30%

Already mentioned above the software does not calibrate the ambient light intensity, only triggers illumination over the telephone. However, this will vary with different phones, as well as different room lighting can cause an error. The next step would be to incorporate a comparison with the color scale on the chip (lighting calibration) into the software. It is also necessary to process software inhomogeneities in the color of the zone, which is not currently processed by the software. Homogeneous color distribution also causes problems, which often occurs on chips itself. To create a homogeneous color distribution, the patches are soaked with a 1% PEG 3000 solution in water prior to application of the reagents. However, this slows down the analysis.

Our AquaChip solution with smartphone evaluation can serve for e.g. as fast on-site water tester in the field. But this research was only proof-of-concept. We are not able to move much further with our university equipment. Above mentioned reagents were used only as proof-of-concept reagents. Theoretically, other reagents that may provide better analytical parameters (LOD, LOQ, linearity, accuracy) can be used to test the parameters. Also, there are only 9 positions of reaction field occupied out of 24 total positions, so it is possible to extend the test with other analysed parameters. We have also tried determination of some other ions

including heavy metals, results are shown in Table 4. So further product direction is possible in the following points:

- Development and testing of better chemical reactions leading to color changes - literature describes reactions reaching better detection limits, respectively in general better analytical parameters, than those used for proof-of-concept - however, these reactions contains specific reagents (including nanomaterial conjugates) that are not commercially/readily available and their costs are high (hence they have not been used in proof-of-concept project), their real potential at reactions on paper is also a question.

- Testing of other reactions for other parameters that could be applied to the "paper tester".

- Development of own tester so that its production price is further reduced.

- Software development for thorough color analysis including small changes and environmental effects (lighting, etc.)

Table 4: Other tested reactions

Analyte	Parameters
Ni^{2+}	30 – 2350 mg/L; error 10 mg/L
Pb^{2+}	100 – 6220 mg/L; error 25 mg/L
Cd^{2+}	60 – 4500 mg/L; error 15 mg/L
Hg^{2+}	100 – 4000 mg/L; error 20 mg/L

Conclusion

The new AquaChip design connects classic paper microfluidic with smartphone detection. The main advantage of the design is the possibility to determine any analyte for which we are able to anchor the reagent onto the filter paper to provide a color reaction. We proved the functionality of our device with basic water analysis/analytes with good analytical parameters, also keeping the production costs low and possibility of the outside-of-the-laboratory use, following our main idea set at the very beginning. There is only the need of storing the finished tests in an airtight container, free from light and moisture. We believe that the AquaChip idea can be further developed from the point of view of the device itself or the software with much wider use.

4.2 TRUE LAB-IN-A-SYRINGE TECHNOLOGY FOR BIOASSAYS

Most of the data presented in this section have been also published in ref. [26]

Chemicals and materials

Standards and reagents, dimethylglyoxime, sodium fluoride, acetic acid, ammonium hydroxide, 1,10-phenanthroline, iron(II) chloride and nickel(II) nitrate hexahydrate, were purchased from Sigma-Aldrich (St. Louis, MO). Methanol for HPLC was purchased from Merck (Darmstadt, Germany). The LIS concept was tested using a model reaction of Ni(II) determination according to the previous works [27,28]. The reagent was prepared as 60 mM solution of dimethylglyoxime in methanol. The blocking agent was prepared as a 1:1 mixture of 0.5 M sodium fluoride and 6.3 M acetic acid (pH 4.5). Finally, the ammonium hydroxide solution (pH 9.5) was used as the reagent buffer. Fe(II) assays was used to study the effect of blocking. Here, 1,10-phenanthroline at concentration 8mg/mL in 6M acetate buffer (pH 4.5) was used as the reagent.

Syringes 2 mL with Luer connection were purchased from Chirana (Stará Turá, Slovakia), Xcut Docrafts puncher with 9.5 mm circle knife was purchased from Artea Trading (Ostrava, Czech Republic), and cellulose fiber sample pads CFSP203000 Millipore were purchased from Labicom (Olomouc, Czech Republic).

Tester fabrication

Two test types were prepared according to the detection part arrangement used in the test. First, the pads were prepared as follows: Millipore cellulose fiber sample pads were cut with the Xcut puncher to rounded pads with diameter of 9.5 mm. The diameter had to be chosen according to the diameter of the syringe barrel (9.0mm our syringes from Chirana). Then the pads were inserted into the syringe, where they formed filtration, conjugation and flow parts of the device. It should be noted that using the same diameters of rounded pads and the syringe barrel (e.g. 9.0 mm pads and 9.0 mm barrel, or 9.5 mm barrel as used in Braun syringes, for 9.5 mm pads) did not fit well. Same pads formed also the detection part in the first type of the test. In second type, detection part was formed by folding rectangular cut pad (27 mm length and 8 mm height) from Millipore cellulose fiber sample into the syringe barrel (see Fig. 20). The pads for conjugation and detection where inserted into conjugation solution or reaction

solution and dried with hairdryer before inserting into the syringe with tweezers. Pads were one-by-one punched to its final form by inserting the plunger to the syringe. The rectangular detection part was rolled up and inserted into the syringe barrel, again by using tweezers. The number of parts for each part of the test depended on the user and the test. Step-by-step fabrication process is displayed in detail on Figure 19.

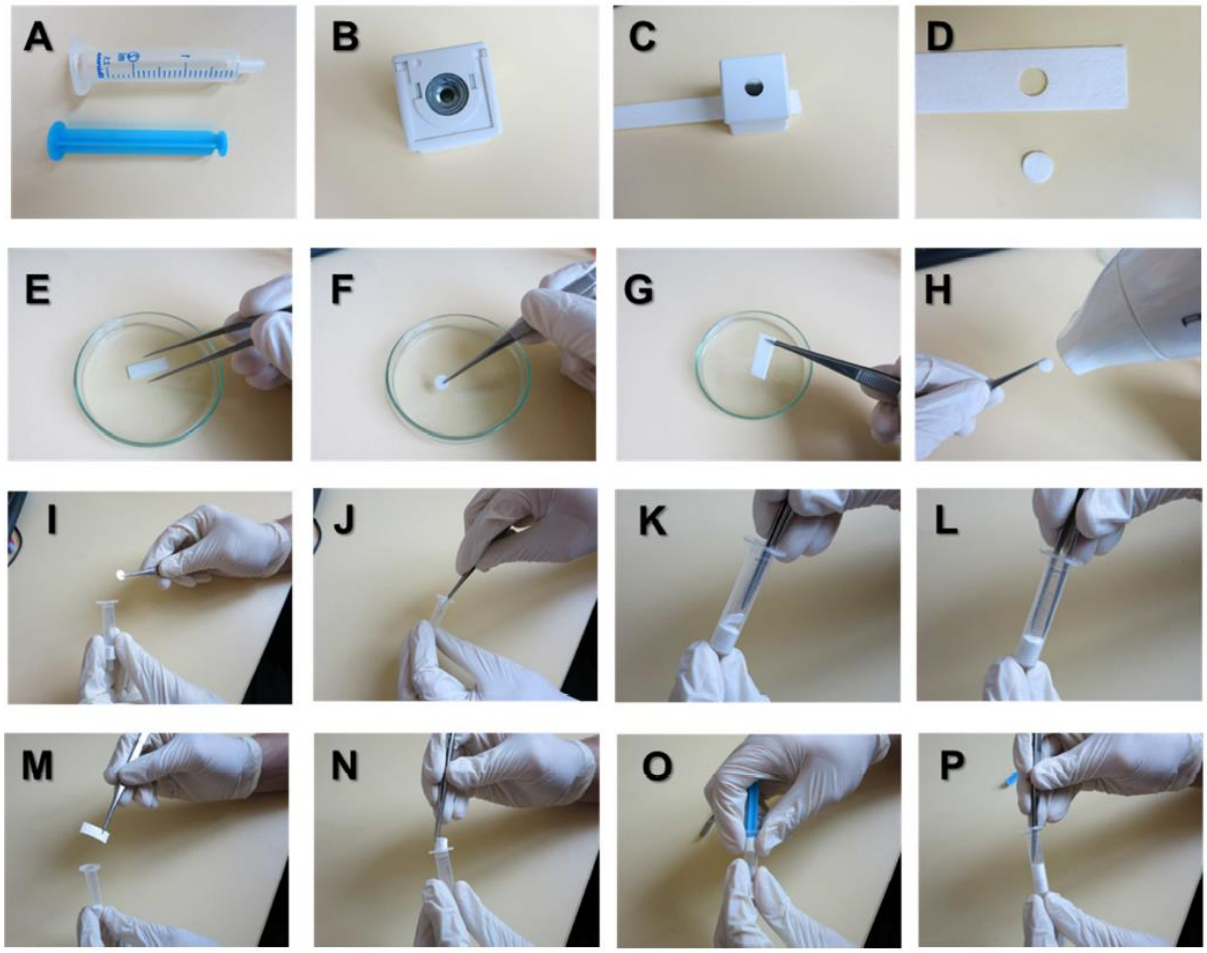

Figure 19. Fabrication process of the lab-in-a-syringe tests

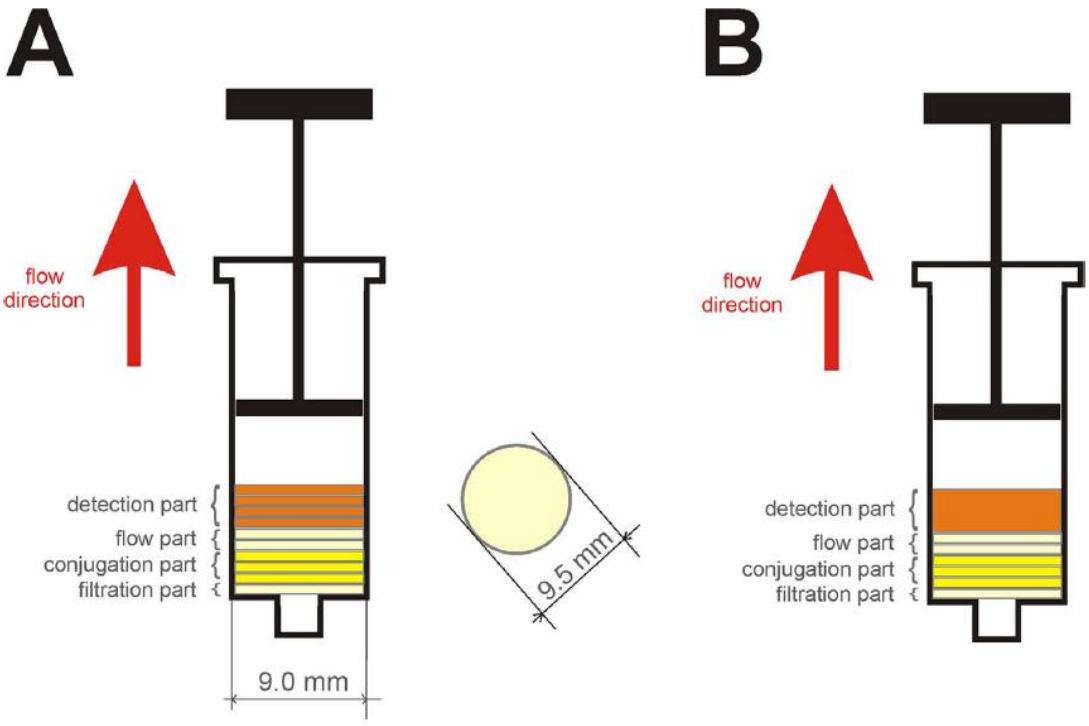

Figure 20. Scheme of the true lab-in-syringe tests. A: LIS test with layered rounded-pads-based detection part, B: LIS test with the rectangular detection part

Detection and processing

After the reaction pads changed its color, we took a photo of the syringes using standard camera Canon PowerShot S110. The image was then transferred into Corel Photo-Paint X4 software. The mask tool was used to pick the colored region and the histogram was then shown. The average value with the standard deviation value was used for calibration. The Ni(II) calibration was done as function of the color intensity calculated as 255 minus the average value on the concentration of the sample.

For testing the blocking agent two limits were determined. First, the intensity of the blank solution, which contained all reagents without Fe(II) was measured, second, the intensity of the non-blocked Fe(II) sample was measured as the 100% intensity. All the other measured intensities were recalculated to percentage using subtraction of the intensity of the blank solution.

Results and discussion

The main problem of the LIS concept in comparison to the classical LFA tests (lateral-flow assays for e.g. common pregnancy test) lays in the detection of the assays.[29] In LFA tests, the detection pad is easily visible for users, but in LIS test, users must open the cartridge which holds the detection pad. This can be dangerous when analysing potentially harmful or infectious samples. We have designed a novel concept of LIS tests, where the conjugation and reaction pads were put directly into the syringe barrel. We have also designed two possible arrangements of the detection part where the detection is user friendly: (A) by using "layered" device where the detection part is composed of a number of rounded pads (five in our case), that increase the thickness of the detection part that needs to be visually analysed, or (B) by using rectangular detection part with an appropriate thickness that is inserted into the barrel (see Fig. 20).

We used classical Ni(II) reaction test with dimethylglyoxime as the reaction agent and the fluorides in acidic environment to block potential cross-reactive species as a model reaction. However, any kind of conjugation solution can be used instead of the blocking agent and also any kind of reaction agent can be used, including traditional immunoassay-based agents. First, we designed the pre-reaction parts of the true LIS test, this is very important mainly for the real samples, where small particles can adsorb conjugation (or blocking agent) or just simply need to be free of undesirable impurities. We used the insertion of one rounded bare part before any other that serves as a primary filter of the reaction solution. Then the conjugation pads were following layer. The number of these pads depends on the amount of the conjugation (blocking) agent reacted. We used Fe(II) as a model compound that needs to be blocked, so the number of pads inserted into the blocking agent should be in correspondence to the amount of covered (blocked) metal. Fe(II) at 1.0 µg/mL were injected into the syringe through 0-5 conjugation (blocking) pads. Quantification of Fe(II) was performed by using classical reaction with 1,10-phenanthroline as described above (we already know this reaction on Millipore cellulose fiber from previous research/chapter). Figure 21 shows, that the amount of blocked Fe(II) slightly increased from 0 to 3 pads, then it reached the acceptable level of 4 or 5 pads. More than 90% of Fe(II) was blocked, so this leading us to the use of 5 pads in the end. This amount ensures blocking of all cross-reactive compounds.

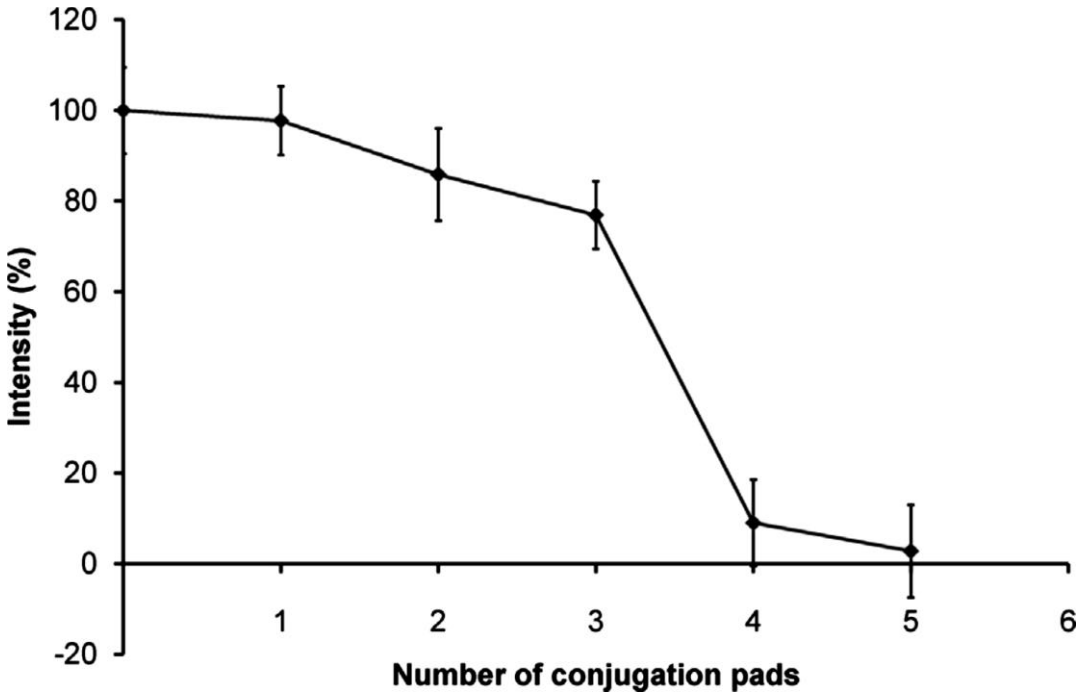

Figure 21. Effect of number of blocking (conjugation) pads on the blocking of Fe(II)

Apart from the main research we studied the effect of insertion of more bare pads between conjugation (blocking) pads and reaction pads. These “flow pads” (as named in Fig. 20) served for the transportation of a sample and for the separation of conjugation and reaction parts. We found that the flow pads were very important for separation of these two tests part, because they prevented leaking of both solutions (also by atmospheric water). In our case, we used three flow pads, as the optimal number. However, flow pads could serve also for separation of potential multiple products and/or products and matrices using paper chromatography principles similarly like in LFA tests. The number of these pads depends on the reaction used in the LIS assay and the associated reagents.

Last aspect of our research was the user-friendliness of the visual detection, which is also the key parameter. We compared two test designs: (A) the layered rounded-pads-based-detection part, and (B) the rectangular detection part. The width of one reaction pad is about 1.5 mm, which makes the test easy to be performed but the visibility is not perfect. Using 5 pads, results in the visible reaction zone having a width of about 7.5 mm, which is fine for common visual detection. Figure 22 shows analysis of different concentrations of Ni(II), clearly showing

the joints of pads being brighter than other places on the pads. But overall evaluation by naked eye estimates the average intensity, by the same principle as the software we used -Corel Photo-Pain. The same test was done with second arrangement using the rectangular detection part with the similar width of 8.0 mm. Results are shown in Figure 23, where can be seen that the suction of the sample into the barrel was more precise in the case of rounded pads-based detection part. This occurred because in rectangular arrangement, the sample liquid could have washed out the precipitate of Ni(II)-dimethylglyoxime complex that hampered the evaluation. Both arrangements provided good linearity in the selected range of concentration with R^2 of 0.998 for rounded-pads detection part and R^2 of 0.931 for rectangular detection part.

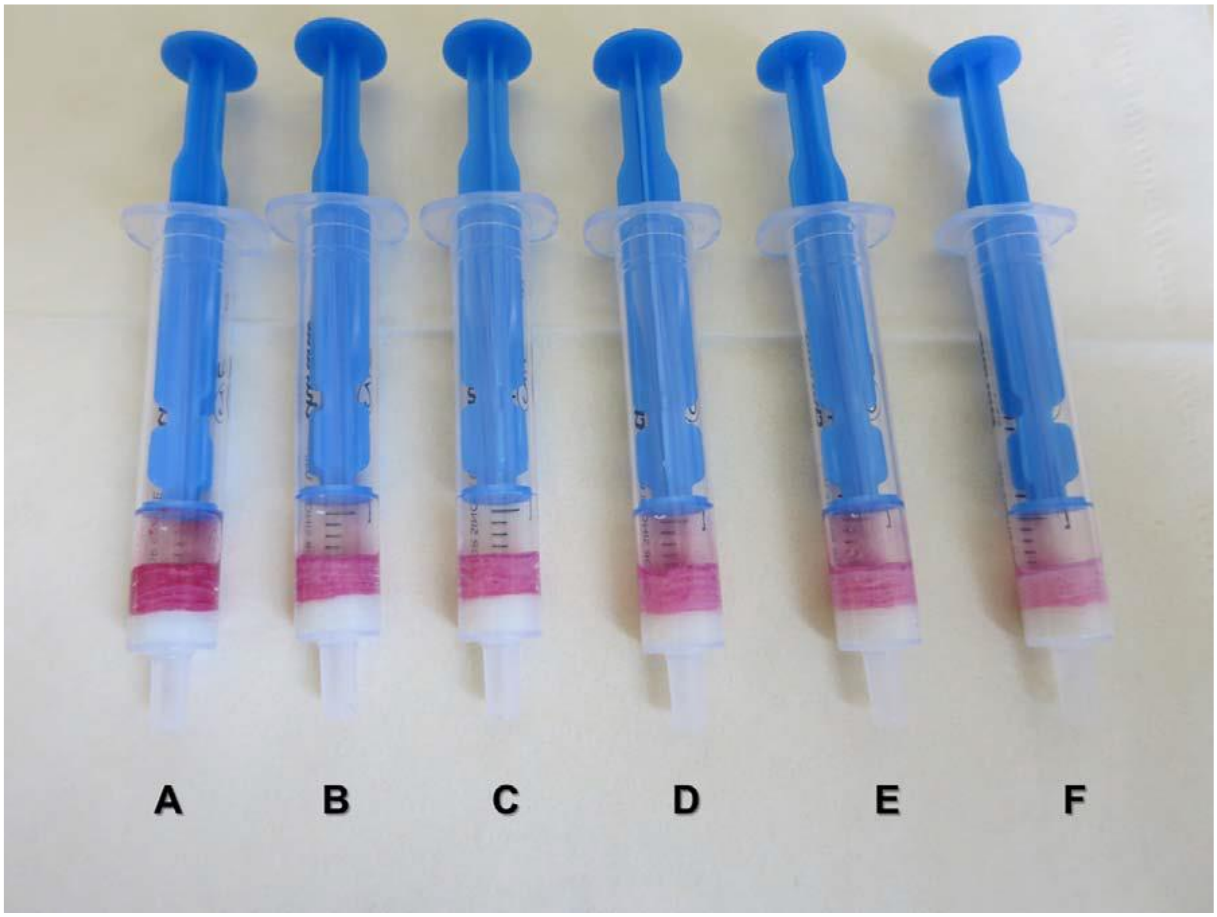

Figure 22. Ni(II) calibration using true LIS test with rounded pads for detection. Ni(II) concentration: A: 0,1 M, B: 0.04 M, C: 0.02 M, D: 0.01 M, E: 0.005M, F: 0.0025M

Figure 23. Photography of Ni(II) calibration using true LIS tests with the rectangular detection part

For user-friendly visual quantification without need of camera, the syringe test can be also enhanced by putting the colored scale onto the barrel. User can easily compare result with this scale, similarly like in pH paper test (see Fig. 24).

Finally, we studied the use of the absorption part over the detection part of the syringe. However, we found that it was not very profitable to use it probably due to the fact that the syringe plunger formed greater flow of the analyzed liquid that could be absorbed by such part.

Figure 24. Syringe barrel with colored scale

Conclusion

In our work we solved a problem of detection in LIS test, by putting all the pads directly into the syringe barrel using more layers to make the results visible for users. It is not necessary to open the cartrige holding the detection part to achieve the detection/quantification. We proved the functionality of our device with determination of Ni(II) levels, but generally the device can be used for analysis including immunoassays because the pads can be divided into conjugation as well as reaction ones. Our true LIS test production costs approx. 2 USD which represents also very low unit price. What is more, there are no specific requirements for storing, as the true LIS tests can be easily stored using classical procedures applied for syringes for medical use. We believe, that the true LIS tests can be very profitable for analytical chemistry in general but they can find use elsewhere.

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CURRICULUM VITAE

PERSONAL DATA

Name: Lenka Hárendarčíková

E-mail: lenka.harendarcikova@upol.cz

EDUCATION

- 2014 – present — Palacký University Olomouc, Czech Republic

  Ph.D. programme: Low-cost microfluidic devices for biologically active agent analysis (supervisor: Dr. Jan Petr)

- 2012 – 2014 — Palacký University Olomouc, Czech Republic

  Master´s degree in Analytical Chemistry

  Master´s thesis: Development of miniaturized low-cost tests for clinical and forensic analysis (supervisor: Dr. Jan Petr)

- 2012 – 2014 — Palacký University Olomouc, Czech Republic

  Bacherol´s degree in Chemistry

  Bacherol´s thesis: Using of spectral methods for analysis of art objects (supervisor: Dr. David Milde)

INTERNSHIPS

- 08– 09/2013

  State Veterinary Institute – Department of Foreign Substances, Olomouc, Czech Republic. **Method validation for ICP-OES determination of feed.**

- 03– 05/2015

  THE BIOS LAB-ON-A-CHIP GROUP, University of Twente, Enschede, Netherlands. **Microfludic DNA separation in PDMS device. (s**upervisor: Prof. Dr. J.C.T. Jan Eijkel)

PROJECTS

- Internal grant agency UP Olomouc, IGA_PrF_2015_020, "Matrix effect and its suppression in sample analysis", member of the research team
- Internal grant agency UP Olomouc, IGA_PrF_ 2016_016, "Bioanalysis and analysis of food", member of the research team
- Technology Agency of the Czech Republic, Partial project of TA CR project TG01010080 Support of technology transfer at Palacký University in Olomouc, „Water quality control: cheap and elegant" (POC_0301), member of the research team
- Internal grant agency UP Olomouc, IGA_PrF_2017_020 „Matrix effect and detection limit", member of the research team
- Internal grant agency UP Olomouc, IGA_PrF_2018_027 „Efficient sample processing with complex matrices", member of the research team
- Internal grant agency UP Olomouc, IGA_PrF_2019_028 „Innovative methods of sample analysis with complex matrices", member of the research team
- Ministry of Education, Youth, and Sports of the Czech Republic, NPU LO 1305 "Development of the center of advanced technologies and materials", member of the research team
- Internal grant agency UP Olomouc, IGA_PrF_2020_030 „Modern trends in analysis of samples with complex matrices", member of the research team

EDUCATIONAL ACTIVITY

- 2015/2016 – ACH/ACC (Cvičení z analytické chemie 6 hours/week)
- 2015/2016 – ACH/CHV (Chemie vody 1 hours/week)
- 2016/2017 – ACH/ACC (Cvičení z analytické chemie 6 hours/week)
- 2016/2017 – ACH/KLCH (Klinická analytická chemie, "microfluidic and medicine" 4 hours)

PUBLISHING ACTIVITY

- **Lenka Hárendarčíková**, Daniel Baron, Andrea Šebestová, Jan Rozsypal, Jan Petr, True lab-in-a-syringe technology for bioassays. Talanta 174 (2017) 285-288.

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CONFERENCE

- Jan Petr, Daniel Baron, Jana Horská, **Lenka Hárendarčíková**, Pavlína Svobodová, Adam Přibylka, Martin Švidrnoch, Vítězslav Maier, Juraj Ševčík, Co-author of the lecture: Capillary Electrophoresis of Nanoparticles: Present and Future Tasks. 4th Annual RCPTM Conference - Devět Skal, 13 - 14 October 2014

- **Lenka Hárendarčíková**, Jan Petr, Poster: Preparation of low-cost microfluidic devices based on filter and bakery papers by using an office laminator. International Interdisciplinary Meeting on Bioanalysis CECE 2014 - Brno, 20 – 22 October 2014

- Martins Rucins, Daniel Baron, **Lenka Hárendarčíková**, Aiva Plotniece, Jan Petr, Poster: Determination of some hormone antagonists by micellar electrokinetic chromatography. International Interdisciplinary Meeting on Bioanalysis CECE 2015 - Brno, 21-23 September 2015

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ANOTHER_ACTIVITIES

- Reviewer of bachelor theses (Pavlína Svobodová, Petra Hubáčková).
- Organizer and regional secretary of the chemistry olympiad (2014 - 2017)
- Organization and participation of popularization events (e.g. Veletrh vědy a výzkumu, Noc vědců, Den otevřených dveří etc.) and school excursions.
- Master Thesis Consultant (Alena Šustková - Development of microfluidic device using 3D print and its testing for forensic purposes)