Microfluidic chip for the sample derivatization prior to mass spectrometry

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MICROFLUIDIC CHIP FOR THE SAMPLE DERIVATIZATION PRIOR TO MASS SPECTROMETRY

Master thesis

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MIKROFLUIDNI ČIP ZA DERIVATIZACIJU UZORKA PRIJE MASENE SPEKTROMETRIJE

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Sveučilišni diplomski studij Fizika, smjer Biofizika

Sažetak:

Za ovaj rad napravljen je mikrofluidni čip koji bi služio za derivatizaciju proteina, te sve kemijske reakcije potrebne za dobivanje rezultata masene spektrometrije. U proteinu bi se vodik zamjenio deuterijem te gledao rezultat pomaka mase. Kod dizajna i pravljenja čipa mora se paziti na faktore kao što su: tokovi, vrijeme reakcije, materijali i metode dostupne za rad, miješanje tekućina itd. U radu će biti izračunate teoretske vrijednosti fizikalnih veličina i opisane metode pravljenja čipa te eksperimentalno testiranje teorijskih veličina. Motivacija za ovaj rad je smanjenje troškova u budućim istraživanja proteina masenom spektrometrijom kombinirana sa zamjena vodika s deuterijem.

Ključne riječi: Mikrofluidi, čip, masena spektrometrija, protein.

Rad sadrži: 35 stranica, 16 slika, 10 tablica, 12 literaturnih navoda. Izvornik je na hrvatskom jeziku

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Master thesis

MICROFLUIDIC CHIP FOR THE SAMPLE DERIVATIZATION PRIOR TO MASS SPECTROMETRY

Ivan Mardešić

University graduate study programme Physics, orientation Biophysics

Abstract: This is a paper on designing and making a microfluidic chip that would be used for hydrogen-deuterium exchange to proteins and after chemical reaction, by mass spectrometry to observe the results of mass spectrometry. There are many factors to making this chip such as flows, reaction times, material, mixing of liquids, equipment available for work, etc. Ultimately three chemical reactions will occur in the chip and at the end of the chip output will be at a given flow for mass spectrometry. The motivation for this work is the automation process and to reduce costs of future research. Automation will improve and accelerate these studies and reduce the errors in protein research, and cheaper research could help make this method available to as many institutes and universities as possible, accelerating the research of protein and hydrogen-deuterium exchange.

Keywords: Microfluidics, chip, protein, mass spectrometry

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Contents

1. Introduction

Microfluids is the area of fluid mechanics where the fluid is kept and manipulated inside channels smaller than millimeter. Usually dimensions of channels range from 10 – 500 μm. Microfluids is a multidisciplinary field where the knowledge of physics, engineering, biology, chemistry is combined. The beginning of micromechanics is associated with the late 1960s, but from 1990s there was more focus researching in microfluids area and applications on different areas of physics and chemistry [1]. The main idea is to design a chip with geometric shape and material to control fluids for different applications. The advantage of microfluidic chips is the automatic process of research and experimentation. Another advantage of microfluidic chips is the very small size of chips, using only milliliters of fluids resulting in low cost testing. It is more practical to use the chips as they do not require a lot of energy and with automatization less error will occur as compared with different methods.

There are many applications of microfluidic chips. Some examples are: chips for genetic analysis, different areas in molecular biology for mass spectrometry, electrophoresis, genomic diagnostic, filters, different biosensors (Antibodies-Based biosensors, Cell-Based biosensors, DNA biosensors), etc. [1]. For all this different application different concepts must be designed for the chip and different materials must be used. For controlling fluids inside channels, controlling units are used; pumps, mixers and valves.

Microfluidic chip in this work is made for sample preparation for mass spectrometry. Protein is used for mass spectrometry and mixed with heavy water (D_2O) to create hydrogen – deuterium exchange (HDX) and to assess mass shift in mass spectrometry. HDX is chemical reaction where hydrogen atom in the molecule is changed with deuterium atom (isotope of hydrogen with one neutron). Prior to mass spectrometry some chemical reactions must happen, and flow needs to be in range (1 - 50μl/min) for ionization method of mass spectrometry to create satisfying results.

Spectra of mass spectrometry is ratio of mass to charge of ions and with spectra we receive information of the molecule of interest.

Objective of this work is developing a new method of HDX in mass spectrometry using microfluidic chip. With the microfluidic chip this method would be automated and easier to use in future. Motivation is the reduced cost of HDX in mass spectrometry, and easier and faster researching than standard methods for HDX.

2. Proteins and Mass Spectrometry

2.1 Proteins

Amino acids are organic molecules that contain amine group (-NH_2), carboxyl group (-COOH) and side chain (-R) that is different for each amino acid. There are 20 [2] amino acids and usually they are divided into 4 groups because of their physical properties of side chains. Those groups are: amino acids with charged side chain (positive and negative), with uncharged side chain, special cases and amino acids with hydrophobic side chain. Amino acids can create a covalent bond with other amino acid, a peptide bond. Peptide bond is covalent bond between carbon of the first amino acid and nitrogen of the second amino acid. In that reaction one molecule of water is released. Figure 1 shows tripeptide.

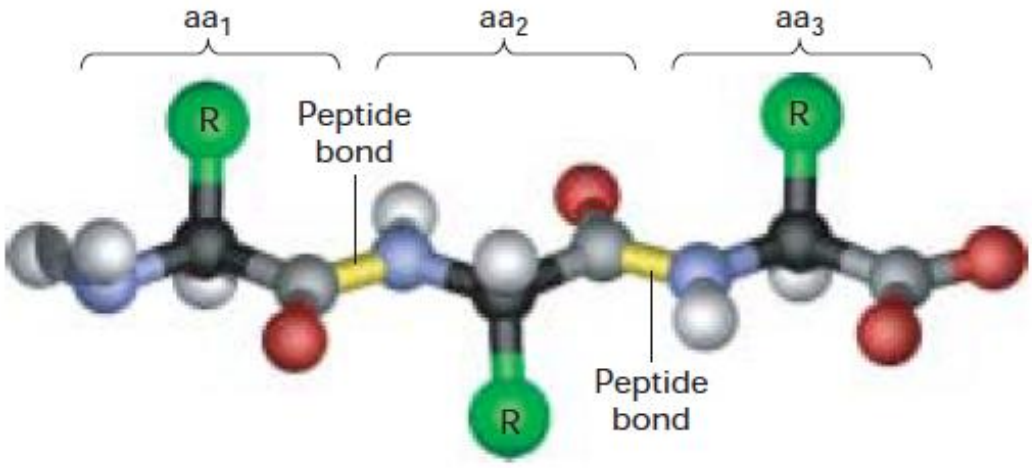

Figure 1 *Peptide bond (yellow) bond that connects carbon atom (gray) and nitrogen atom (blue). aa stands for amino acid. Side chain R (green) largely determine properties of protein. Figure taken from [2].*

When more amino acids form peptide bond, the polypeptide is form. Combinations of polypeptide form a protein, large biomolecule. Proteins are very important for living organisms. All main functions in cells are made by proteins like catalytic reactions, DNA replication, building material for cells, transporting molecules etc. Structure of proteins is divided into 4 groups: primary, secondary, tertiary and quaternary structure. Primary structure is amino acid sequence, secondary structure local structure like α helix and β sheet. Tertiary structure is the folding of the protein and making 3D structure subunit and quaternary structure forms more protein subunit making single protein complex [11]. Figure 2 a) shows all structure group of protein, and figure 2 b) shows functions of protein.

The research of proteins is rapidly advancing, and many methods focus on researching proteins. The most popular methods for discovering the structure of protein are: x-ray crystallography, nuclear magnetic resonance, mass spectrometry and electron microscopy. Each method has advantages and disadvantages; however, the combination of all methods can provide us with information about the structure and conformational change of proteins. After finding the structure of the protein (position of every atom of protein), software is used to make a simulation of protein kinetics in organism to investigate their interaction with other molecules. In this work there will focus on mass spectrometry, advantages and disadvantages of that method and hydrogen – deuterium exchange in mass spectrometry.

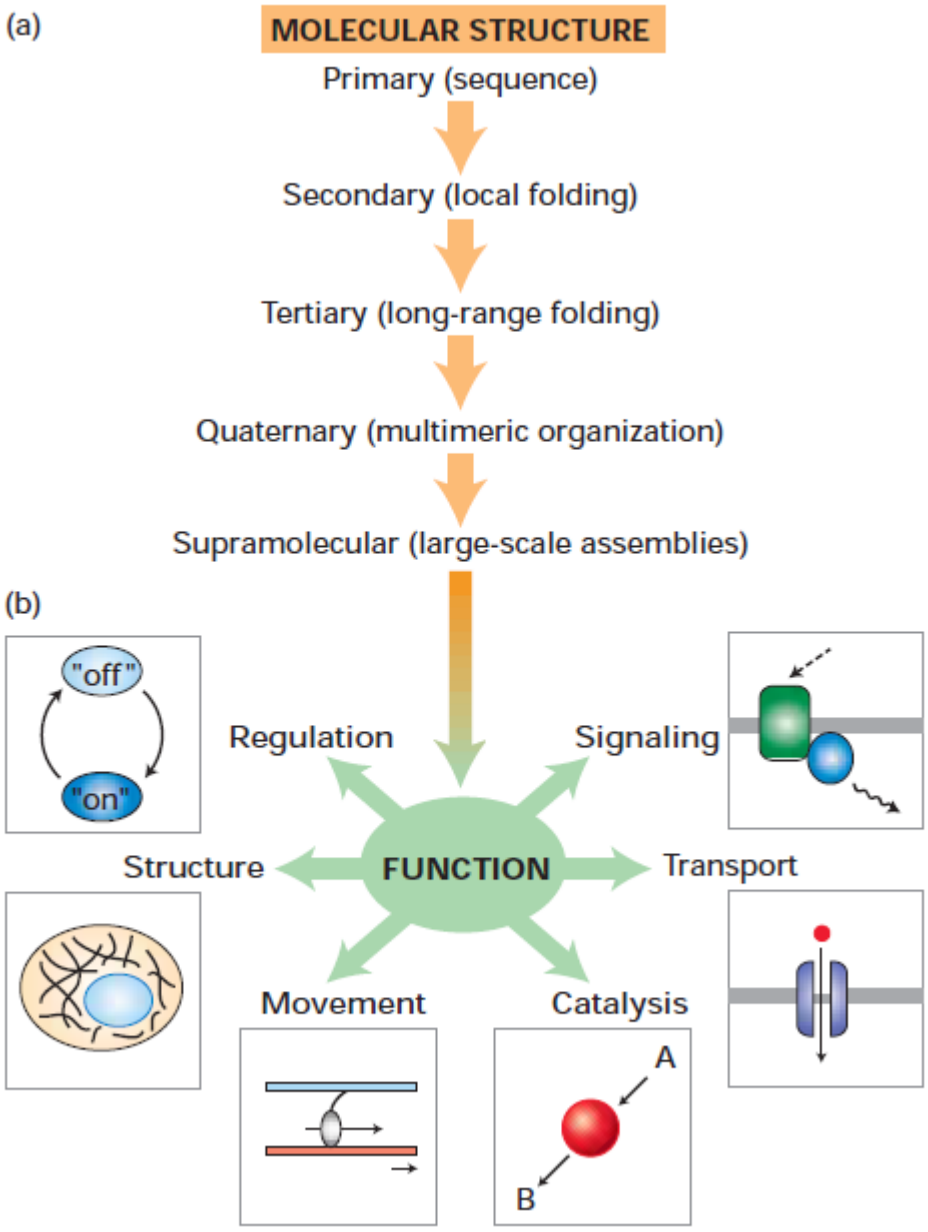

Figure 2. *Overview of structure and function of protein. a) Primary structure (linear polypeptide) folds into secondary structure (α-helix or β-sheet) that packs in tertiary structure (globular domain). More globular domains form quaternary domain, and some protein have tens to hundreds subunits (supramolecular). b) Functions of protein that can be signaling, regulation, catalysis, transport, movement, structure of cell. Figure taken from [2].*

2.2 Mass spectrometry and Hydrogen – Deuterium Exchange

Mass spectrometry is a technique for measuring basic properties of molecules. It can be a chemical molecule or macromolecule like protein. It measures mass to charge ratio. In the beginning of mass spectrometry only small chemical molecules were measured, but later there were two ionization methods developed: Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) which allowed biological macromolecules measurement possible. In this work, ESI is available for ionization of protein. electrospray ionization is method that creates ions by dispersing liquid stream into droplets using high voltage at liquid [3]. Droplets are firstly reduced in size, then droplets fracture or molecular ions are released due to charge density exceeding surface tension of the droplet.

Mass spectrometry for proteomic can be functional or structural. For functional proteomic, proteins are digested with pepsin into peptides, a method called “bottom-up” analysis. When the protein is intact that method is called “top-down” analysis. “Top-down” analysis is used for the structural research of proteins, while “bottom-up” is used for functional research of protein. The method for this study will be “bottom-up” analysis.

Three components of mass spectrometry are: ion source, mass analyzer and ion detection system. Components are in vacuum and in that vacuum depending of mass spectrometer, it can be from $1 \cdot 10^{-3}$ to $1 \cdot 10^{-9}$ torr [3]. In this case electrospray ionization is ion source, then charged solvent droplets are transported in mass analyzer with electrostatic lenses.

In 1989 Wolfgang Paul got Nobel Prize in Physics for contributing for developing quadrupole mass analyzer [3]. It consists of four parallel rods. For pair of opposite rods direct current and radio frequency is applied. Only ions with stable trajectories for given direct current and radio frequency will pass rods, other ions will collide with them. Ion trap mass analyzer have in addition charged end caps that allows trapping of ions. This mass analyzer is used in biology because they do not require ultra-low vacuum and they are used with electrospray ionization. Quadrupole and ion trap analyzer are basic, but there are more developed methods used for mass analyzers.

Ion detection system can be different: dynode electron multipliers (EM), multichannel plates (MCP) and Fourier transform method. Each detection system depends on the mass analyzer.

Dynode electron multipliers is a vacuum tube. It transforms primary emission of ions hitting dynode electron multipliers into secondary emissions of electrons. Current is proportional of numbers of ions hitting dynode electron multipliers. Dynode electron multipliers is used with ion trap and quadrupole mass analyzers.

HDX for proteins is a chemical reaction exchange of main chain amide hydrogen with deuterium. HDX depends on amino acids surrounding, global location of protein and solvent properties. HDX is catalyzed by both acids and base, therefore the reaction rate is the sum of acid and base rate:

$$k_{ch} = k_{int,A}[H_3O^+] + k_{int,B}[OH^-] \quad (1)$$

where k_{ch} is unprotected HDX rate, and $k_{int,A}$ and $k_{int,B}$ catalyzed reaction constant of acid and base [3]. Minimum HDX rate is when pH is between 2 and 3 and temperature is 0 °C [3].

Following model is used to explain HDX occurring in more events. The first event is the separation of H-bonds due to the dynamics of proteins. It is the change in protein before denaturation or because of solvent conditions. After the first event, amide hydrogens are accessible to solvent and can be exchanged with deuterium. Hydrogen deuterium exchange is a slow reaction, however, hydrogen inside of protein exchange is significantly slower than hydrogens accessible to solvent. Depending on the structure of the protein, some hydrogen must make contact with the solvent and that requires conformational change caused by dynamics of proteins. Figure 3 shows a schematic view of a small area of proteins unfolding upon HDX and refolding again with a consistent rate of k_1 and k_{-1} that represents folding and refolding and k_2 rate of HDX.

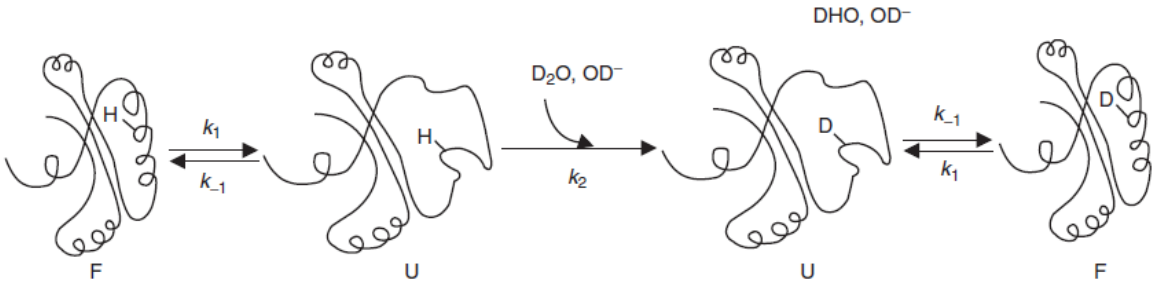

Figure 3. *Small region of protein folded (F) goes in unfolded (U) conformational change, then HDX can occur and later that region is again folded.* k_1 *and* k_{-1} *are reaction rates of folding / refolding of protein and* k_2 *is HDX rate. Figure taken from [3].*

There are two exchange mechanisms that depend on constant rates. They are named EX1 and EX2. If k_{-1} is much higher than k_2 ($k_{-1} >> k_2$), folding must occur multiple times to create the exchange. This exchange mechanism is called EX2. If $k_2 >> k_{-1}$ leads to unimolecular exchange EX1.

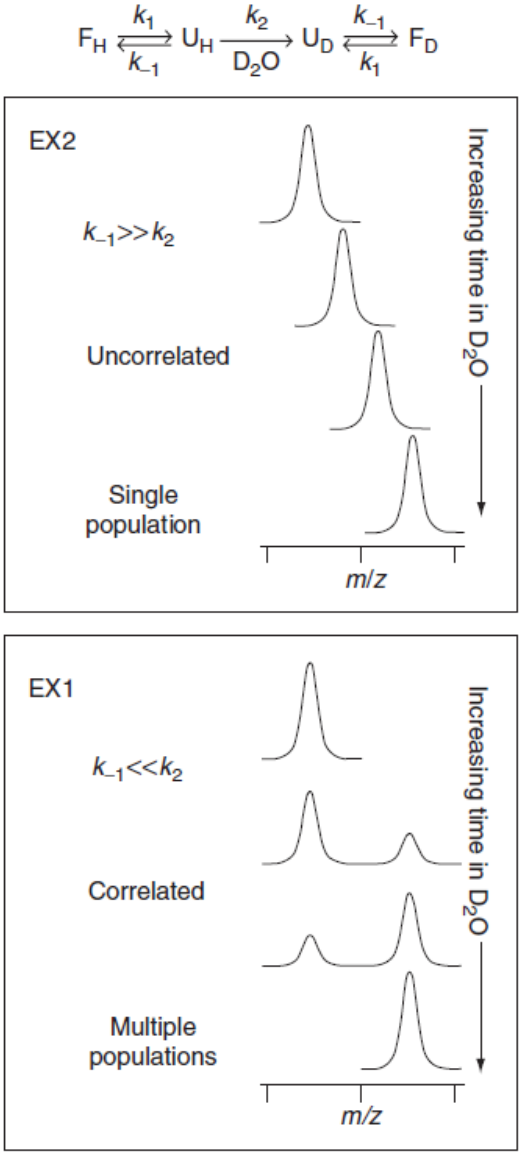

Figure 4. *Mass spectrometry of protein deuterium exchange for two different exchange mechanism. Figure taken from [3].*

HDX experiment has to occur in a series of steps. First the protein is mixed with the solvent of D_2O in a ratio D_2O to H_2O is 20 to 1. The second step is stopping the reaction by changing the pH level 2 and change the temperature, T = 0 °C. The final step is to enter mass spectrometry analysis and determine peptides that have mass shift.

HDX – mass spectroscopy has advantages because some proteins cannot be studied with x-ray crystallography or with electron microscope, because some proteins are hard to isolate and make crystal structure of protein, while with HDX we got new information of protein depending of mass shift. There are some disadvantages of this method. HDX is very sensitive on pH of solution. The main problem is also the back exchange when deuterium is exchanged back with hydrogen atom and this is the reason why we require the step of stopping reaction. One of the biggest disadvantages of HDX without microfluidic chip is the long time required to complete the measurement. However, microfluidic chip measurement will be in order of minutes.

3. Materials and methods

3.1 Materials

There are different types of materials that can be used for microfluidic chips, mainly the most popular polymers: cyclic olefin copolymer, (COC), poly (methyl methacrylate), PMMA, polycarbonate (PC), polyethylene terephthalate (PET), polyester and poly(dimethylsiloxane), (PDMS). Each material has advantages and disadvantages depending on the application of the microfluidic chip. Materials have different optical properties, micromachined properties, chemical properties like compatibility with the buffers or compatibility with cells and thermal and electrical properties [4].

Material chosen for microfluidic chip for mass spectrometry is polycarbonate (Figure 5.). Reasons for this are because of the ability to easily manufacture channels with a milling machine, compatibility with buffers that are needed prior to mass spectrometry and low price of PC.

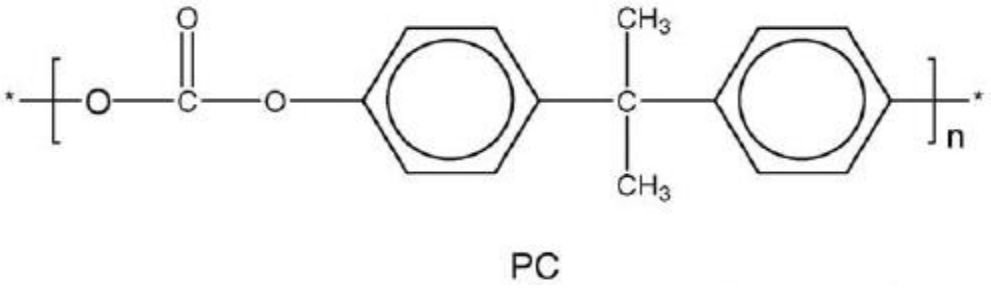

Figure 5. *Monomer unit of polycarbonate. Figure taken from [4].*

For connecting pumps with microfluidic chip, tubes are needed. First silicon tubes are tested with dimensions of 0.5 mm inner diameter and 2.1 mm outer diameter. Silicone tubes were selected because of their low price.

Microfluidic chip was manufactured for testing silicone tubes, then tubes were glued on and tested with water.

Silicon tubes were tested successfully because no leakage occurred, however, later in research the tubes that are going to be used are Ethylene tetrafluoroethylene material (ETFE) or IUPAC name poly(1,1,2,2-tetrafluorobutane-1,4-diyl), because their outer diameter is 1.6 mm which make them easier to use with type of pumps available for work.

3.2 Methods

For manufacturing microfluidic chip, CNC (Computer Numerical Control) milling machine was used. The G-code programming language is controlling the CNC machine. Inside the CNC machine, a 10 x 10 cm plate can be fitted. Milling head for milling channels on plate is 300 μm of diameter. Milling head for milling holes for tubes on polycarbonate is 1.6 mm diameter. Figure 6. shows the CNC machine used in this research. Operating the CNC machine was done by a CNC machine operator. Design of microfluidic chip was made by me which the CNC operator manufactured.

Figure 6. *CNC milling machine while milling polycarbonate plates.*

Two polycarbonate plates are milled to make final microfluidic chip. On top, the side holes for tubes are drilled. After both plates are milled, they are connected in a vacuum chamber on high temperature (135 ℃). Two holes were drilled on same position on both polycarbonate plates for connecting it precisely one over other.

After microfluidic chip is manufactured the last step is gluing tubes inside holes. After gluing the tubes inside microfluidic chip, it takes 24 hours for glue to harden and then the microfluidic chip is ready for use.

4. Micromixer

4.1 Micromixer theory

Within the microfluidic chip, there are usually more than one flow with different substances that need to be mixed to create a chemical reaction. Because of regime of laminar flow in microchannels, diffusion is main force of mixing, and long channels are required for sufficient diffusion time [5]. Due to the fluids not mixing in short microchannels, a micromixer must be placed inside of the microfluidic chip. There are two different types of micromixers; active and passive. Active micromixers use external power like electrokinetics, magnetohydrodynamics, magnetic force and acoustic force [1]. They are more expensive, so the focus in this work will be on passive micromixers. Passive micromixers are channels with geometrical shapes made for mixing fluids, and they do not require external power. Most popular passive micromixers are: mixers in shape of letters X, Y, T, split and recombination mixers, tortuous mixer, herringbone mixer etc. Micromixer activity is depending of Reynold number and some of them are easier to manufacture than others because of their geometric shapes. For high Reynold number flow is turbulent, and for low Reynold number flow is laminar. At laminar flows, vicious force is dominant, and for the turbulent flows, dominant forces are inertial forces.

Split and recombination micromixer is tested and used in the microfluidic chip for HDX. The idea of split and recombination mixer is to do a geometrical shape of the channel, so the fluid is divided into two flows and recombined later, then split again and so on [6].

Figure 7 shows schematic view of serpentine laminating micromixer split (SLM). In Figure 8 numerical simulation and difference between serpentine laminating micromixer split and T type mixer for 50 μL/min. Serpentine laminating micromixer split micromixer is better than T type at low Reynolds numbers.

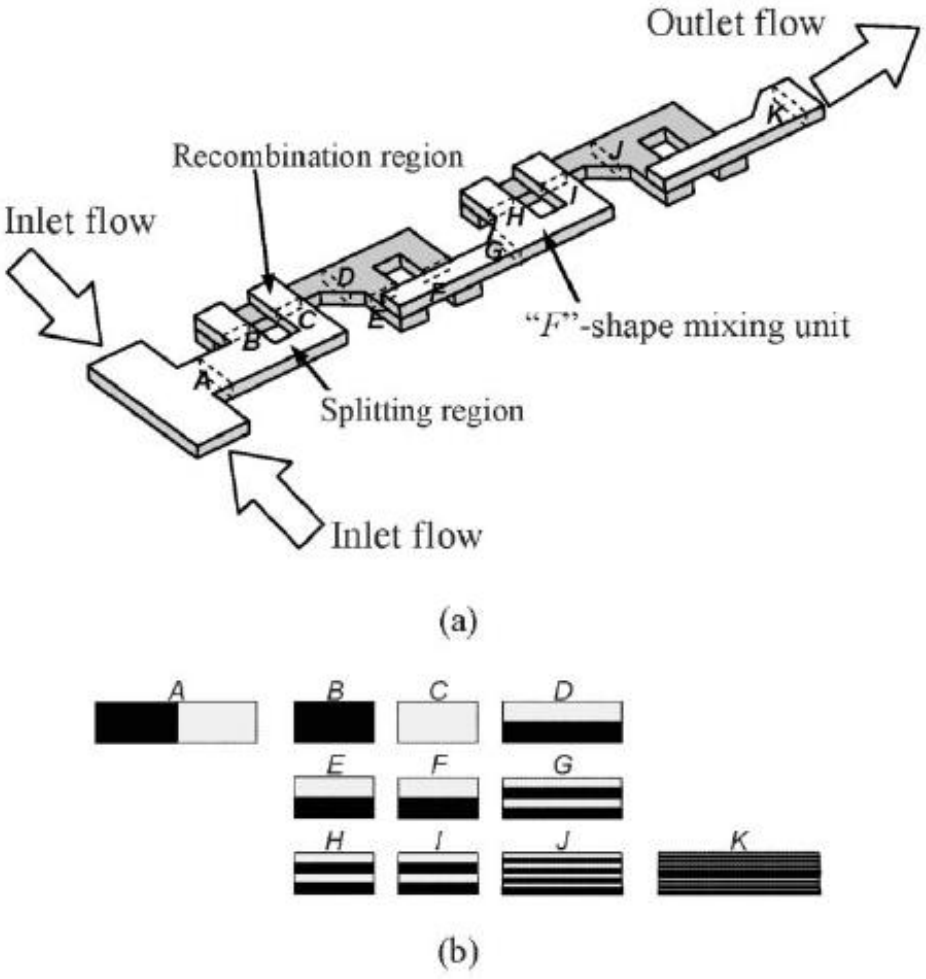

Figure 7. *A) schematic view of serpentine laminating micromixer. B) cross-sectional behaviors for two ideal fluids. Figure taken from [7].*

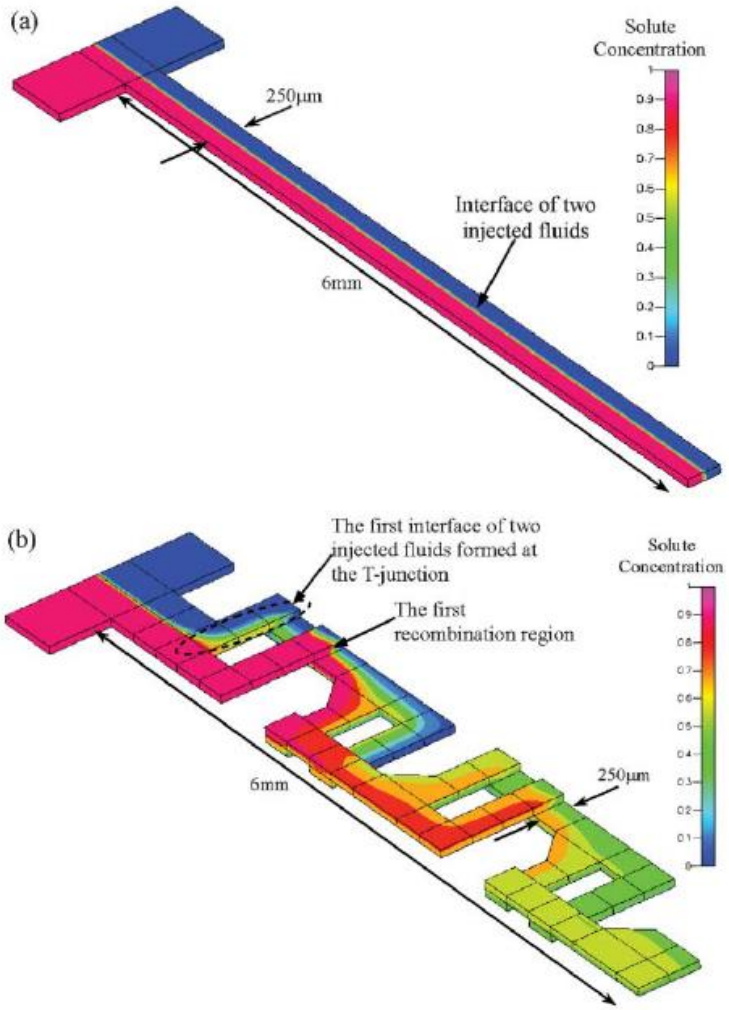

***Figure 8**. Numerical simulation for (a) T type micromixer and (b) and serpentine laminating micromixer for flow of 50 μL/min. Figure taken from [7].*

Another example of split and recombination mixer is XH or XO. Their geometrical shape looks like X where they separate and H, where they recombine. Figure 8 shows numerical simulations of concentration of fluids in straight channel and XH mixer at Reynold number at Re = 0.56.

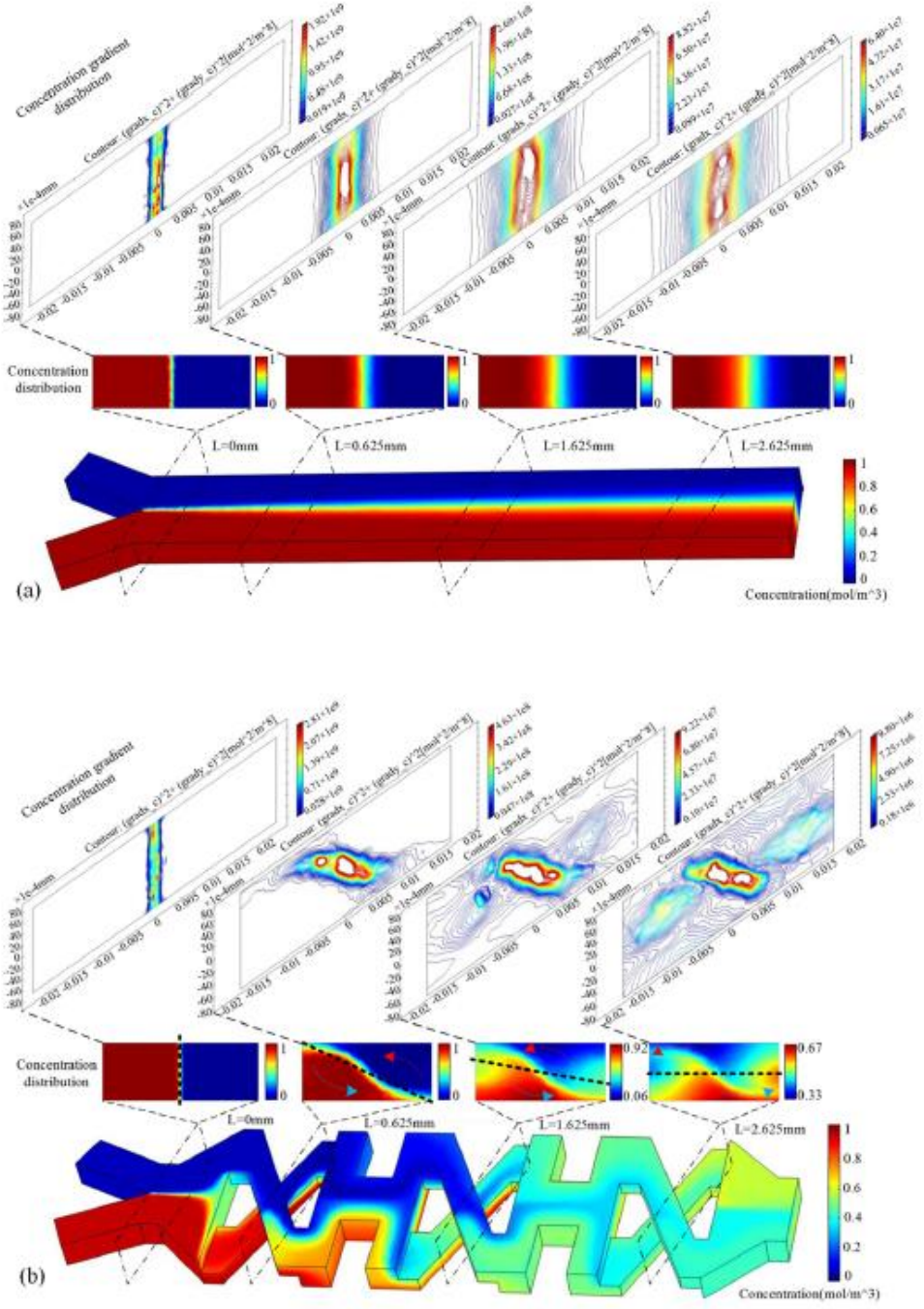

Figure 9. *Compare of numerical simulations of (a) straight channel and (b) XH mixer. Re = 0.563. Figure taken from [6].*

Figure 10 shows the half cycle of the herringbone mixer, another group of passive micromixers. Split and recombination mixer is taken for testing in this research, because it is easier for the milling machine to manufacture split and recombination mixers than herringbone mixers.

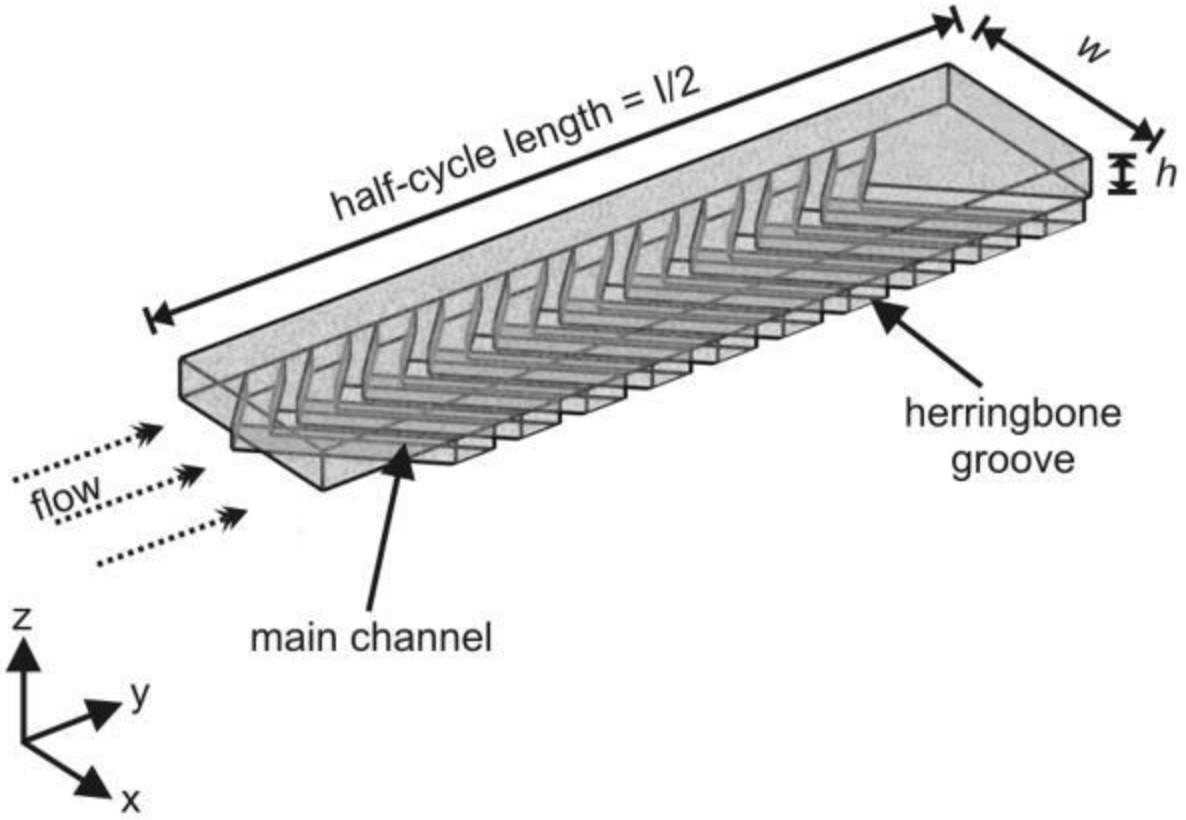

Figure 10. *Half-cycle of herringbone micromixer with area of w · h with 10 herringbone grooves. Figure taken from [8].*

4.2 Testing of micromixer

For testing the micromixer, serpentine laminating micromixer was manufactured. Width and height of channels are 300 μm. For testing, formic acid (CH_2O_2) was used at pH=3 and phenol red indicator ($C_{19}H_{14}O_5S$) with NaOH pH=11. Phenol red is a purple color at pH = 11 while formic acid is colorless. If those fluids are mixed, the color of the fluid will be yellow. Figure 11. shows serpentine laminating micromixer split with phenol red and formic acid. After the third split and recombination the fluid turns yellow. This shows that the micromixer is working like the numerical simulations in Figure 8. Pressure applied from pumps was 100 mbar.

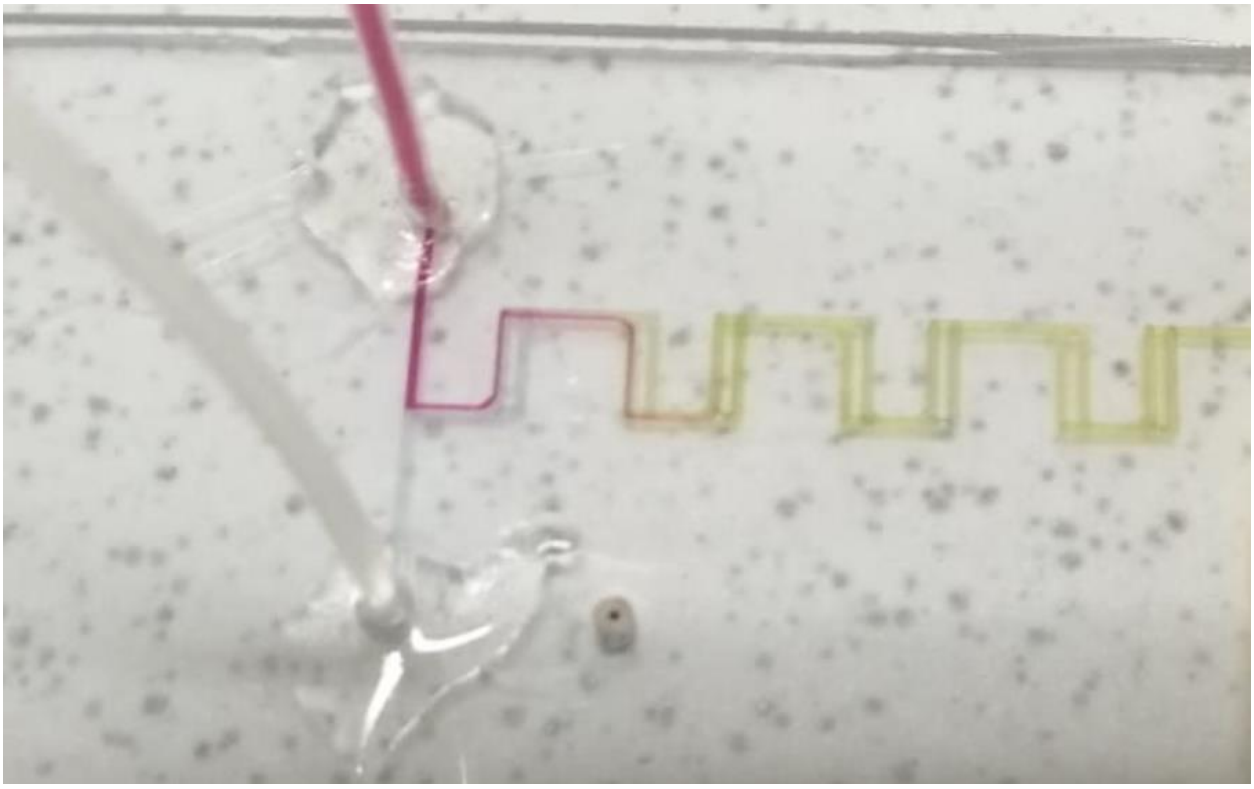

Figure 11. *Split and recombination serpentine laminating micromixer split. One channel is filled with phenol red and second channel is filled with formic acid. Yellow fluid is mixture of phenol red and formic acid.*

Figure 12. shows the microchip with two XH split and recombination micromixer is completed in other research done by college at ISAT. The same chemicals were used to test XH micromixer like the F-type micromixer and the same pressure was applied. In figure 11 similar results can be seen like the numerical simulations in figure 9.

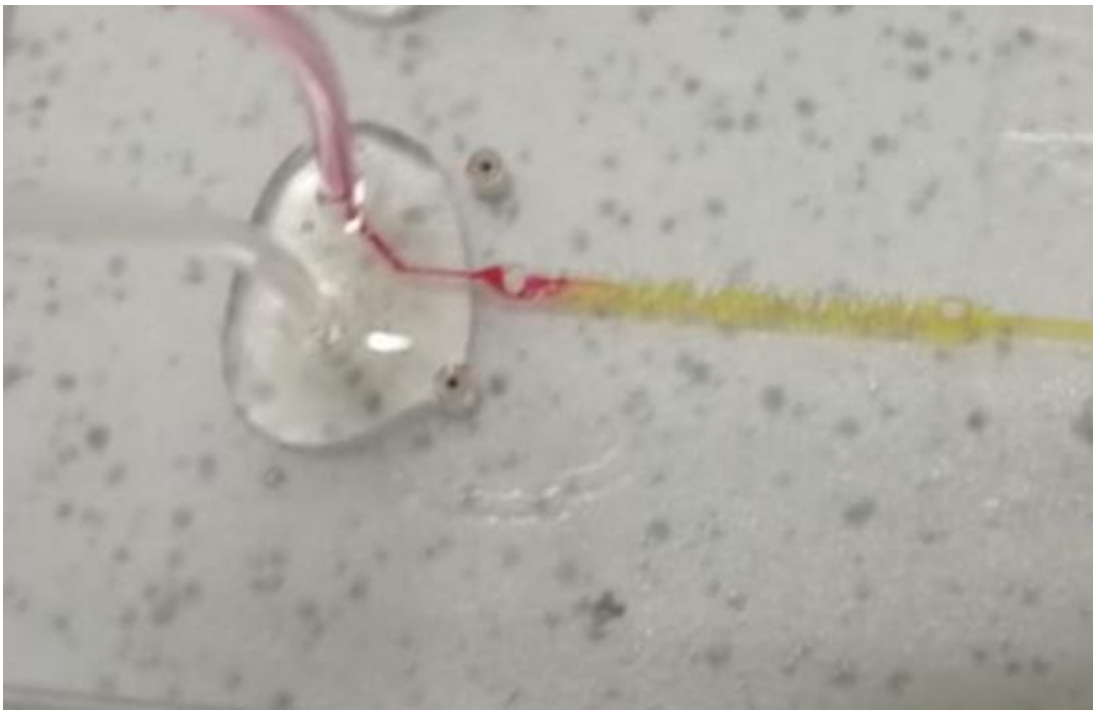

Figure 12. *Split and recombination XH type micromixer. One channel is filled with phenol red and second channel is filled with formic acid. Yellow fluid is mixture of phenol red and formic acid.*

5. Calculations and design of microfluidic chip

5.1 Calculations of channels

Before designing microfluidic chip calculations of channels had to be made.

Because the range of flow for electrospray ionization is 1 – 50 µl/min dimensions of channels had to be calculated so output flow is not bigger than 50 µl/min.
In microfluidic chips, rectangular channels with dimensions width (*w*), height (*h*) and length (*l*) are milled.

Equation that connects pressure and flow is:

$$\Delta p = R\rho\dot{V} \tag{2}$$

where *R* is hydraulic resistance, ρ density of fluid and $\dot{V}$ flow of fluid [9].

Equation (2) is analog with Ohm's law where Δp represents voltage, *R* represents resistance and $\dot{V}$ electric current.

For calculating hydraulic resistance equation was used [10]:

$$R = \frac{2\eta}{\rho}\left(4.7 + 19.64\ \frac{1+\left(\frac{w}{h}\right)^2}{\left(1+\frac{w}{h}\right)^2}\right)\frac{(w+h)^2 l}{4w^3h^3} \tag{3}$$

From flowrate and volume of channel resistance time can be calculated with equation:

$$t_r = \frac{V}{\dot{V}} \tag{4}$$

Resistance time is how long it takes for fluid to fill the channel. With resistance time, average velocity of fluid is calculated using equation:

$$v = \frac{l}{t} \tag{5}$$

Reynold number is dimensionless number that is used in fluid mechanics to predict the flow of the fluid. It is ratio between inertial to viscous forces. At low Reynolds number flow is laminar, and at big Reynolds number flow is turbulent. In turbulent flow fluid's speed and direction is changing. Laminar flow is when *Re* < 2300. Reynolds number has wide applications: Making Navier-Stokes equation dimensionless, for aerodynamics engineers, for solid objects at fluids, etc.

Reynolds number is calculated with equation [11]:

$$Re = \frac{\rho\, v\, d}{\eta} \tag{6}$$

where *d* is hydraulic diameter, η is viscosity of fluid. η and ρ are used for D_2O are $\eta = 1.25$ mPa·s and ρ = 1100kg/m^3 [12].

For calculation of hydraulic diameter of rectangular channel equation is used:

$$d = \frac{4\,A}{P} \tag{7}$$

where *A* is area and *P* is wetted perimeter of the cross-section

$$A = w\,h \tag{8}$$

and

$$P = 2w + 2h \tag{9}$$

For calculation of tubes different equations are used. For volume of circle shape tube equation used is:

$$V = r^2\, l\, \pi \tag{10}$$

where *r* is inner radius of tube and *l* is length of tube [6]. Resistance for circular channel is:

$$R = \frac{128\,\eta\,l}{\rho\,\pi\,d^4} \tag{11}$$

Where *d* is inner diameter of channel.

Diffusion time is also approximated for the micromixer [9],

$$t_d = \frac{w^2}{4D} \tag{12}$$

where *D* is diffusion constant. For protein it is $D = 10^{-10}\ m^2/s$ [5].

5.2 Design of microfluidic chip

There were some main factors that need to be considered before designing the microfluidic chip. For HDX there are 3 chemical reactions before going on mass spectrometry. First is D_2O reaction with protein(aq) and that reaction will occur in range 30 s to 5 minutes. Flows are in 20:1 ratio. The second chemical reaction is adding TCEP (tris(2-carboxyethyl)phosphine), urea, formic acid and pepsin. In this chemical reaction, protein is denatured. For this step flow of denaturation chemicals, it does not matter, but the chemical reaction must occur within the 1 – 5 minute range. Final step is adding HCN in flow ratio 1:1. This chemical reaction happens very fast, and this reaction is needed for ESI.

Five holes must be drilled in which tubes can be glued, four for input and one for output. Three mixers are needed because of three chemical reaction that inside microfluid chip. SLM is chosen for microfluid chip because it was tested, and it is easy to manufacture on CMC machine.

The width of channel is $w = 300$ μm, because the diameter of the milling head is 300 μm, height chosen for channels are $h = 400$ μm and inside mixer $h = 200$ μm.

Microfluid chip can be separated in 10 flow units (Figure 13).

Channel 1 is input channel for protein, channel 2 is input channel for D_2O and channel 3 is first serpentine laminating micromixer split. On figure 13 is a schematic view of bottom side of chip

so only four split and recombination units can be seen, and another 4 are on the top side of the chip. Channel 4 is designed so that more times passes before the reaction occurs. Channel 5 is the input of TCEP/urea/Formic acid/pepsin, and channel 6 is the second micromixer. Channel 7 is the same reason as channel 4 for allowing enough time for the reaction to occur. Channel 8 is the input of hydrogen cyanide (HCN) and channel 9 is the third micromixer. Last channel 10 is the output for electrospray ionization.

On the top side of the chip, only 4 split and recombination units were milled for 3 micromixers (Figure 13.). With a 1.6 mm diameter milling head, holes for tubes were drilled.

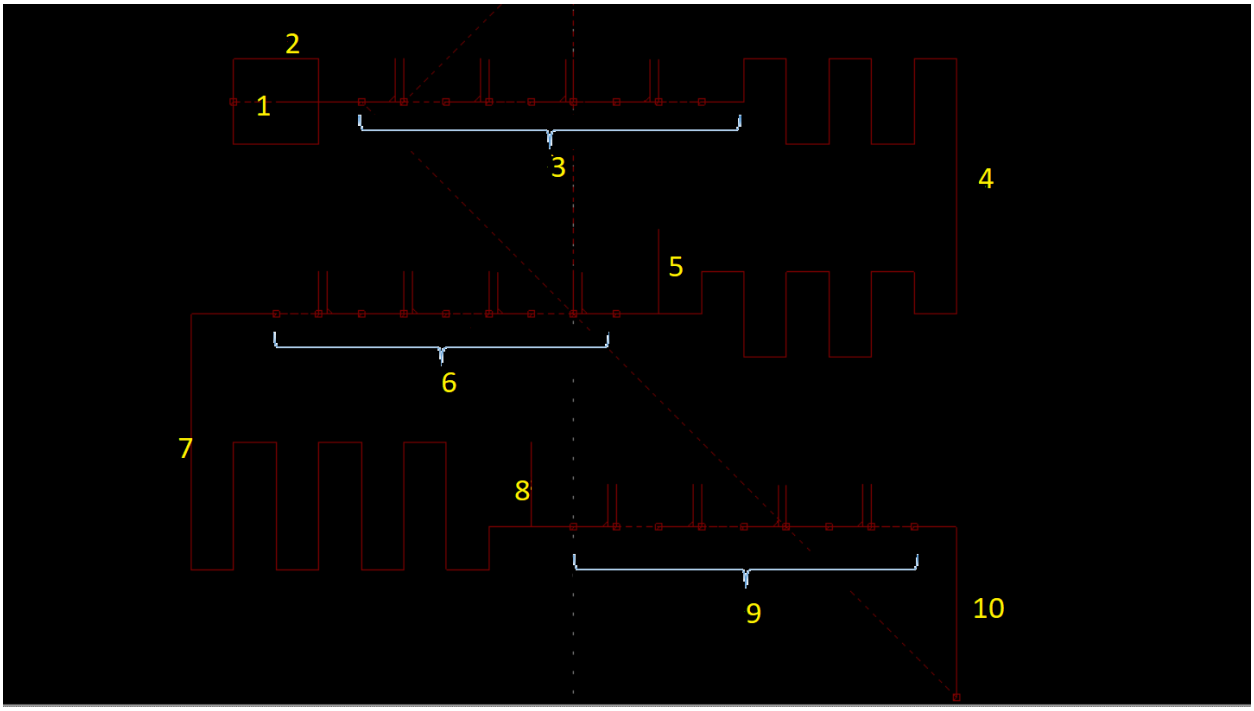

Figure 13. *Schematic view of bottom side of microfluidic chip. Channel 1 is input for protein, channel 2 input for D_2O, channel 3 is first serpentine laminating micromixer split, channel 4 is for mixture of D_2O and protein, channel 5 is input for TCEP, urea, pepsin, formic acid. Channel 6 is second serpentine laminating micromixer split, channel 7 is for resistance time for stopping reaction, channel 8 is input for hydrogen cyanide, channel 9 is third serpentine laminating micromixer split and channel 10 is output.*

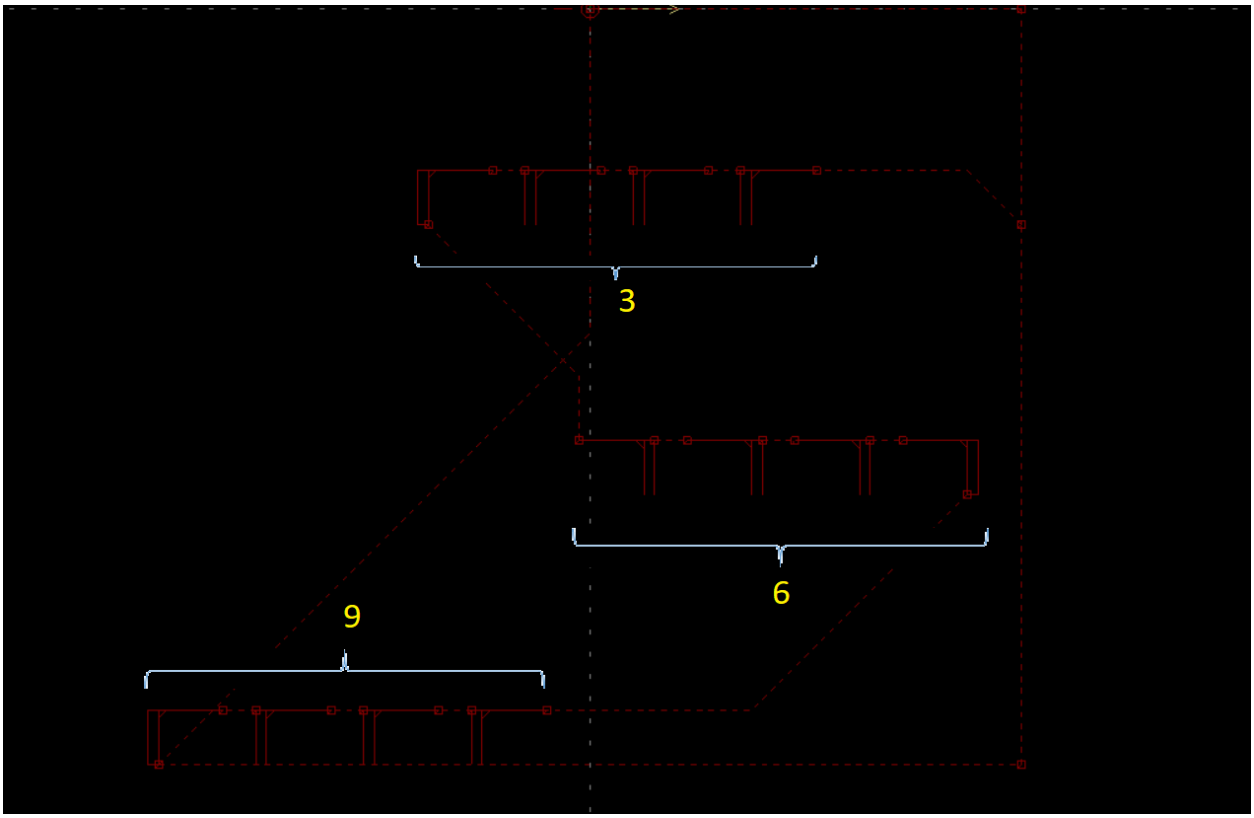

***Figure 14**. Schematic view of top side of microfluidic chip. Channel 3 is first serpentine laminating micromixer split, channel 6 is second serpentine laminating micromixer split and channel 9 is third serpentine laminating micromixer split.*

After choosing dimensions of channels, the next input is choosing flow for channel 1 of protein and choosing the flow of channel 5 with TCEP/urea/Formic acid/pepsin. In table 1 physical parameters for flow of 0.7 μL/min inside channel 1. Back pressure was calculated using equations (2) and (3), Reynold number is calculated using equations (5) and (6). Resistance time is calculated using equation (4).

Table 1. Physical parameters for channel 1 for flow of 0.7 μL/min

Q_1	0.70	μL/min
w_1	0.0003	m
h_1	0.0004	m
l_1	0.005	m
V_1	$6.00 \cdot 10^{-10}$	m^3
$t_{r\ 1}$	51.43	s
p_1	0.2	Pa
Re_1	$3.70 \cdot 10^{-2}$	

In table 2. are shown physical parameters for channel 4. The most important parameter for channel 4 is resistance time, because the duration must be long enough for chemical reaction to occur. Because flow in channel 1 is 0.7 μL/min, channel 2 is 14 μL/min and flow inside channel 4 is sum of Q_1 and Q_2. In tables we see that in both channels Reynold number is less than 1 and inside all channels of microfluidic chip is laminar flow. Resistance time for channel 4 is 97.96 s, which is inside the range of 1-5 minutes.

Flow in channel 10 for Q_1 = 0.7 μL/min Q_4 = 3 is Q_{10} = 35.6 μL/min which is in range of ESI.

All other tables are shown in appendix.

Table 2. *Physical parameter for channel 4.*

Q_4	14.70	μL/min
w_4	0.0003	m
h_4	0.0004	m
l_4	0.2	m
V_4	$2.40 \cdot 10^{-8}$	m^3
$t_{r\ 4}$	97.96	s
p_4	243	Pa
Re_4	0.61	

6. Results

For testing the microfluidic chip four pumps were needed. There were two pumps working, and other pumps did not work because of the problem with software. Because of this problem alternative method was used in work. One method for testing microfluidic chip was to use hydrostatic pressure:

$$p = \rho\, g\, h \tag{13}$$

Where g is gravity constant and h is height of water.

In tables there are back pressure of channels so total pressure difference was calculated to see which pressure each pump should have.

Pressure at pump 1 for pumping protein is p_1 = 2798 Pa. Pressure at pump 2 for pumping D_2O is p_2 = 2818 Pa. Pressure at pump 3 for pumping acids and pepsin is p_3 = 2025 Pa, and pressure at pump 4 for hydrogen cyanide is p_4 = 1150 Pa.

Using equation (13) the heights were calculated. For pump 1 and 2 height of water must be h_1 = 29 cm, for pump 3 height is h_2 = 21 cm, and for pump 4 h_3 = 12 cm.

Test was unsuccessful because the water did not enter the chip. In figure 15. is shown how test was done, using water with blue dye.

Figure 15*. Testing microfluidic chip using hydrostatic pressure. The water was used with blue dye.*

Second alternative method was using syringes to calculate the flow form syringes and then test theoretical calculations of resistance time. To calculate flow the scissor elevator platform was used (Figure 16.). Elevator have rotation button for lifting. To calculate flow, volume of water that goes out of syringe per one rotation of elevator was calculated. Per one rotation, 550 μL of water leaves syringe. For testing of microfluidic chip, flow was 275 μL/min. For doing that, half of rotation was done in 1 minute.

For testing only input for protein and D_2O was used and to calculate resistance time of channels 1, 2, 3 and 4.

Theoretical resistance times are: $t_{r\ 1}$ = 0.13 s, $t_{r\ 2}$ = 1.91 s, $t_{r\ 3}$ = 0.63 and $t_{r\ 4}$ = 2.62 s.

Total resistance time for first four channels is: t_r = 5.29 s.

For experimental work resistance time is $t_{r\ exp}$ = 8.68 s.

A lot of error can happen to this experimental setup, because experiment was done by hands. First is very hard to have same continuing flow, and to rotate button in half cycle in 1 minute. Second is to noticed water entering chip and notice when it enters channel 5 and then press stopwatch. Another problem is that flow for HDX are 100 times less than made in this experiment, so possibility for error was much greater. Because of this reasons error happen, but order of theoretical calculations is correct.

Figure 16. *Experimental setup for testing theoretical calculation of microfluidic chip.*

7. Conclusion

In this work microfluidic chip for mass spectrometry and HDX is presented. There can be much more research about this microfluidic chip in future, first testing with pumps to test all theoretical calculations. After testing theoretical calculation, it can be connected with mass spectrometry and tested is microfluidic chip gives good results on mass spectrometry.

To develop more efficient chip, pepsin solid state can be added to reduce costs of experiment even more. If the air bubbles enter chip, before chip it can been added bubble trap to trap all the bubbles to enter chip.

Those are one of many future researches that can be done on this area of microfluidics. Motivation for this is to reduce costs as much as possible of mass spectrometry researches with HDX.

8. Literature

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9. Appendix

9.1 Tables

Table 3. *Parameters for channel 2*

Q_2	14	µL/min
w_2	0.0003	m
h_2	0.0004	m
l_2	0.045	m
V_2	$2.4 \cdot 10^{-9}$	m^3
$t_{r\,2}$	0.10	s
p_2	11.55	Pa
Re_2	62	

Table 4. *Parameters for channel 3*

Q_3	14.7	µL/min
w_3	0.0003	m
h_3	0.0002	m
l_3	0.096	m
V_3	$5.76 \cdot 10^{-9}$	m^3
$t_{r\,3}$	23.5	s
p_3	420	Pa
Re_3	0.86	

Table 5*. Parameters for channel 5*

Q_5	3	µL/min
w_5	0.0003	m
h_5	0.0004	m
l_5	0.01	m
V_5	$1.2 \cdot 10^{-9}$	m^3
$t_{r\,5}$	24	s
p_5		Pa
Re_5		

Table 6. *Parameters for channel 6*

Q_6	17.7	µL/min
w_6	0.0003	m
h_6	0.0002	m
l_6	0.096	m
V_6	$5.76 \cdot 10^{-9}$	m^3
$t_{r\,6}$	19.53	s
p_6	505	Pa
Re_6	1.04	

Table 7. *Parameters for channel 7*

Q_7	17.7	µL/min
w_7	0.0003	m
h_7	0.0004	m
l_7	0.18	m
V_7	$2.2 \cdot 10^{-8}$	m^3
$t_{r\,7}$	73.2	s
p_7	263	Pa
Re_7	0.74	

Table 8. *Parameters for channel 8*

Q_8	17.7	µL/min
w_8	0.0003	m
h_8	0.0004	m
l_8	0.01	m
V_8	$1.2 \cdot 10^{-9}$	m^3
$t_{r\,8}$	4	s
p_8	14.6	Pa
Re_8	4.83	

Table 9. *Parameters for channel 9*

Q_9	35.4	µL/min
w_9	0.0003	m
h_9	0.0002	m
l_9	0.096	m
V_9	$5.76 \cdot 10^{-9}$	m^3
$t_{r\,9}$	9.76	s
p_9	827	Pa
Re_9	1.76	

Table 10*. Parameters for channel 10*

Q_{10}	35.4	µL/min
w_{10}	0.0003	m
h_{10}	0.0004	m
l_{10}	0.03	m
V_{10}	$3.6 \cdot 10^{-9}$	m^3
$t_{r\,9}$	5.08	s
p_9	59.7	Pa
Re_9	1.25	