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d-LIVER

ICT-enabled, cellular artificial liver system incorporating personalized patient management and support

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OBJECTIVE: ICT-2011.5.1

D4.4: Report on Fluidic Modules for Blood Biochemistry Cartridge

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Document Main Author(s): Tobias Schunck (FhG-ICT-IMM)

Document signed off by: Professor Calum McNeil (UNEW)

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1. Executive Summary

The state-of-the-art techniques for liver patients involve time-consuming consultations at least every few days. At present, there is no instrument for regular at-home monitoring in liver patient management. Because of its patient-friendliness and its user-optimised handling, the d LIVER system will be able to fill this gap. The system will measure six blood parameters (clotting time, Na^+, K^+, bilirubin, albumin, and creatinine) at home on a regular, possibly daily, basis and will consist of the blood biochemistry instrument (BBI) and the blood biochemistry cartridge (BBC). Patients will be able to insert the cartridges and measure his parameters themselves and the comfort for the patient will be increased by the fact, that the measurement will be carried out in the home environment without any waiting time. Patients will be able to test themselves on a regular basis and thus increase the number of results. With more results, the patient can be much better monitored. The d-LIVER system will manage the interaction between patients and clinicians, based on the measured parameters. Therefore, patients will only have to attend clinics when absolutely necessary.

In this deliverable the microfluidic modules included in the BBC are reported. The development and the functionality of the different microfluidic modules are described. Firstly, the function of the blood sampling module for introducing the blood into the cartridge is proven. Secondly, the blood clotting and the serum extraction is tested positive in the serum generation module. The last new microfluidic development is the dilution module. Thirdly, the combination of all three microfluidic modules in a pre-final test cartridge is demonstrated. Finally, the development of the reservoir chip, which is necessary for separating liquid from dry storage, is reported.

At a project meeting on February 12th and 13th 2014, the current status with respect to the fluidic pathway, liquid mixtures and volumes, and instrumental limitations was discussed by WP3 (sensors), WP4 (microfluidics), and WP6 (instrumentation). In addition, the general sensor types were agreed upon.

2. Introduction

This deliverable reports on the different microfluidic modules developed in the d-LIVER project. These modules guarantee the stable and reliable functionality of the microfluidic blood biochemistry cartridge (BBC). The BBC is a disposable part to be used by a liver patient at home and will be operated by the blood biochemistry instrument (BBI). The system consisting of the BBI and the BBC will measure six blood parameters (clotting time, Na^+, K^+, bilirubin, albumin, and creatinine) on a regular, sometimes daily, basis. From the viewpoint of the patient, the use of the BBC should be as easy as possible within the home environment and with minimal potential for harm. For these reasons, the fundamental design rule is the minimization of the volume of blood to be provided to the BBC by the patient, such that a standard finger pricking technique can be used.

This report is divided into two main sections: (1) the development process of the microfluidic modules integrated into the BBC, and (2) designing the reservoir chip for the storage of the liquid reagents.

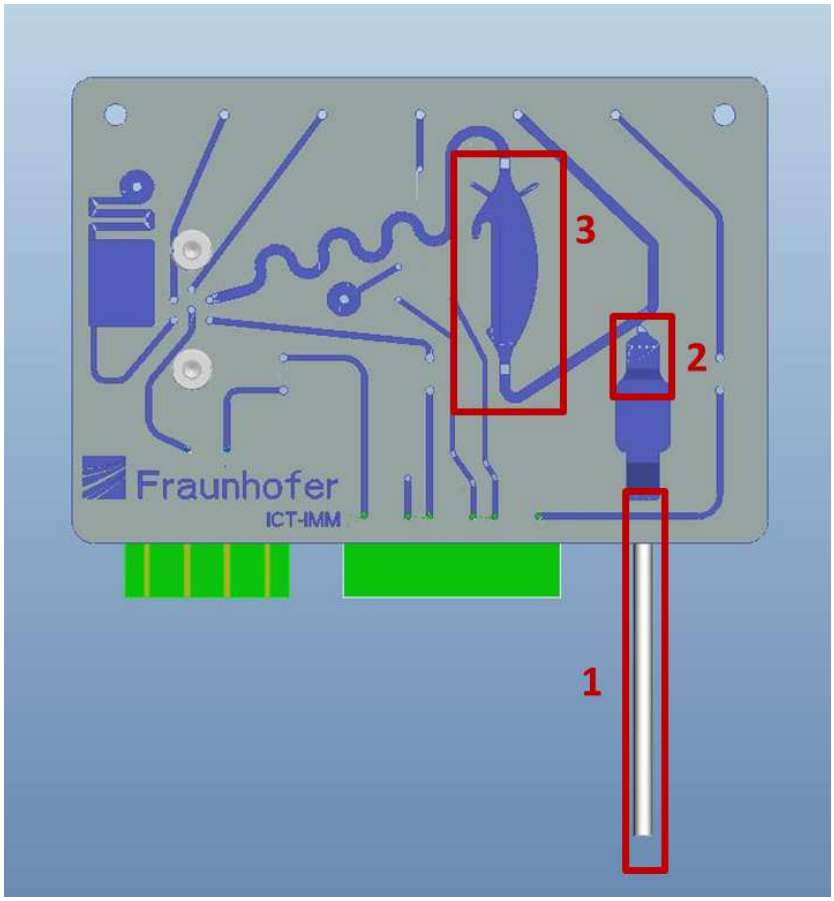

Figure 1: Pre-final microfluidic cartridge with microfluidic modules highlighted in red. 1: blood sampling; 2: blood clotting and serum extraction; 3: liquid dilution.

Since this a Public deliverable, some information has had to be withheld from certain sections of this report due to confidentiality. This information will be separately communicated to the Commission and its reviewers.

3. Microfluidic modules in the blood biochemistry cartridge (BBC)

3.1. Blood sampling

Experiments addressing the feasibility of the blood sampling were performed. As decided, small volumes of blood (10 - 30 µl) should be taken by the patient and introduced into the cartridge via a capillary which is integrated into the cartridge.

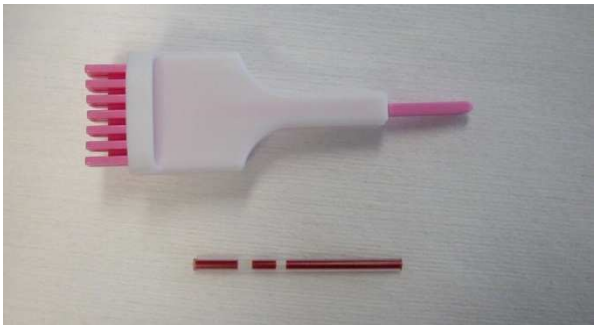

Figure 2: Disposable device for finger pricking and an incompletely filled capillary

For blood sampling the patient has to prick their finger with a commercially available and disposable finger pricking device. FhG-ICT-IMM tested various types of finger pricking devices – an example is shown in Figure 2**Error! Reference source not found.** – and no significant differences between the devices were observed. In the next step, the patient has to gently squeeze their finger to press out a droplet of blood which should remain on the fingertip. The droplet is then brought in contact with the end of the blood sampling capillary and once contact is established, the blood is sucked into the capillary by capillary forces. If the patient disconnects the droplet from the capillary before it is completely filled, the blood plug in the capillary does not move further as long as the capillary is in a horizontal position. To completely fill the capillary, the patient can again provide a droplet to the end of the capillary. If the capillary is not in a horizontal position, subsequent steps of sampling may lead to air inclusion in the blood plug (e.g. Figure 2). The tests led to the following design rules for the BBC and the blood sampling:

- The capillary has to be in a horizontal position during blood sampling.
- The back-end of the capillary needs to be vented to avoid a build-up of pressure during the blood sampling. A build-up of pressure would counteract the capillary forces and lead to an incompletely filled capillary.
- The patient can repeatedly present a droplet of blood to the end of the capillary and easily judge by eye if the capillary is completely filled.
- The end of the capillary inside the cartridge should be “free” (in the sense that the blood plug does not connect to the microfluidic channel) to avoid uncontrolled capillary transport of the blood-plug into the cartridge.

The functionality of an under-pressure-actuated transport of blood from the capillary into the cartridge was shown experimentally. A pressure difference below 10 mbar was necessary. Typically, an under-pressure of approximately 4 mbar completely sucked the blood into the cartridge (Figure 3).

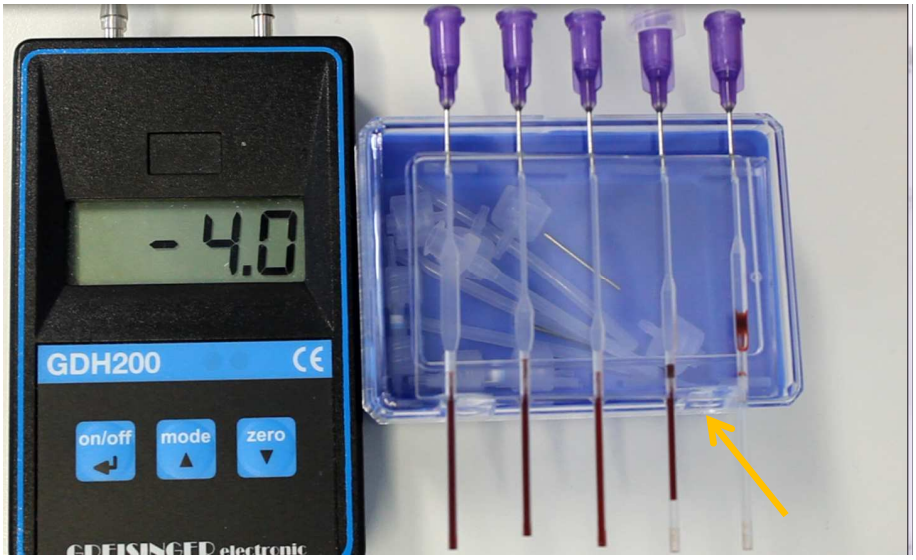

Figure 3: Pressure difference to transfer the blood from the filled capillary into the cartridge (arrow).

3.2. Serum generation

The first steps in the whole process are the clotting and the clotting time measurement. For further measurements, serum has to be extracted from blood. If the clotting time measurement and the serum extraction can be performed in a single step, the whole microfluidic procedure would be tremendously simplified. Therefore, FhG-ICT-IMM addressed the serum generation after clotting as one of the major issues.

For the clotting time measurement, FhG-ICT-IMM realized a special chamber where the clotting takes place. The measurement of the prothrombin time (PTT) requires the coagulation measurement in the presence of thromboplastin. To realize the long term storage of thromboplastin, FhG-ICT-IMM employed a freeze drying protocol. Thromboplastin pre-stored in the chamber triggered the clotting reaction as soon as the blood was transported into the chamber and simultaneously initiated clotting time measurements. After clotting, under-pressure was applied to the stable blood clot, and the serum was sucked out of the clot.

Initial results demonstrated that it was possible to extract 10 µl of serum from the 20 µl of blood. However, this contained a high number of remaining red blood cells (RBC) and thus a filtration step might be essential or the chamber design would have to be further optimized. After several tests with optimized chambers, the results suggested the possibility of serum extraction without any filter membrane.

In the latest chamber design (Figure 4) an under-pressure of 50 mbar was applied for serum extraction. Stop structures supported the stability of the blood clot and decreased the number of RBCs in the serum. In subsequent experiments FhG-ICT-IMM focussed on the quality of the serum concerning the haemolysis and the remaining RBCs.

To this end, the on-chip serum was compared with the serum prepared by blood centrifugation, which is the laboratory standard procedure to generate serum (2000g; 10 min). First, on-chip serum generation and serum made by centrifugation were compared microscopically. Both sera seem to be comparable at first sight (Figure 4).

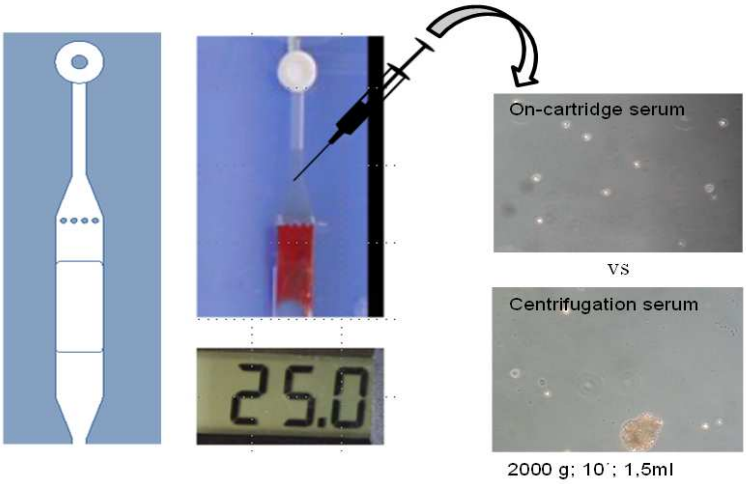

Figure 4: Comparison of sera. Microscope pictures of on-cartridge serum without filtration and centrifuged serum.

FhG-ICT-IMM started to investigate the haemolysis of the RBC during processing. To determine the haemolysis during the extraction process, both types of serum were compared spectroscopically using a nanodrop8000 machine. Haemoglobin shows a prominent peak in the spectrum, the so called Soret band. The centrifuged serum showed less absorption at 415 nm (Soret band) than the on-cartridge serum. Centrifuged serum showed an absorption of 1.1 whereas the on-cartridge showed an absorption of 2.6 (Figure 5).

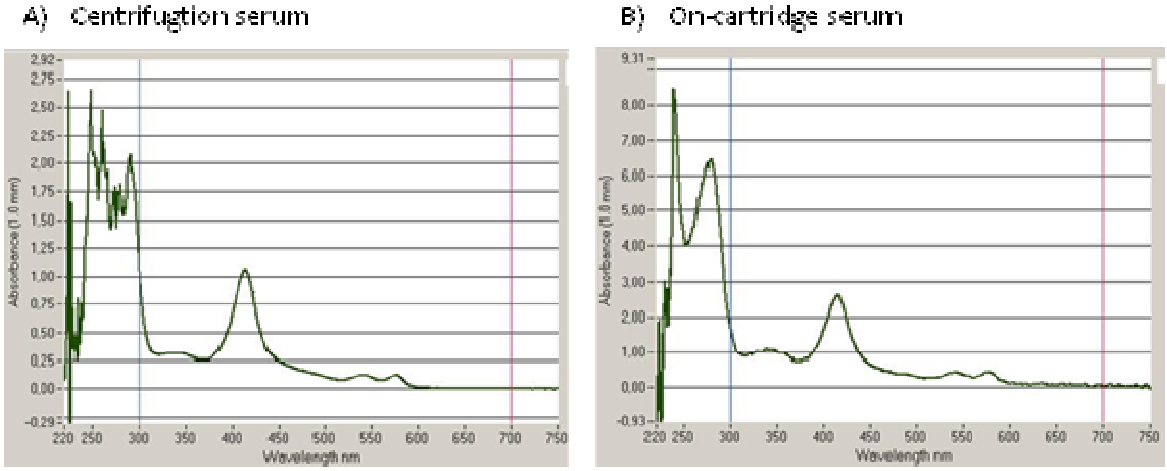

Figure 5: Comparison of centrifuged serum vs. on-cartridge serum. The spectra were recorded with a nanodrop8000 machine. Centrifuged serum showed an absorption value of 1.08 whereas the on-cartridge serum showed an absorption value of 2.67.

Due to the fact that natural haemolysis always takes place in the body, in further experiments the additional haemolysis occurring during the blood sampling and the serum extraction process were determined. FhG-ICT-IMM calculated the percentage of additional haemolysis (Equation 1) using the percentage haemolytic ratio r_h based on absorption rates according to Dey & Ray [2003]. These spectroscopic measurements focus on a wavelength of 545 nm. Centrifugation was used to remove the intact cells from the solution. Serum prepared from untreated blood was used as a negative control whereas completely lysed blood was used as a positive control.

Equation 1: Calculation of the percentage haemolytic ratio from the absorption values.

$$r_h = 100\frac{A_{\text{sample}} - A_{\text{negative control}}}{A_{\text{positive control}} - A_{\text{negative control}}}$$

The intracellular concentration of potassium is huge (approx. 30 times higher) compared with the extracellular concentration. Therefore, haemolysis had a tremendous impact on the potassium concentration in the extracted serum.

While investigating the haemolysis, all blood processing steps had to be tested. The first "blood" steps are the finger pricking and blood sampling. To minimise the impact of sampling errors by the patient during blood sampling, the blood lysis during this step was investigated. The most frequent mistake was to squeeze the fingertip during blood sampling. As highlighted in Table 1, the increase of the haemolysis is visible but negligible.

Table 1: Additional haemolysis and thus additional potassium induced by incorrect procedure (squeezing the finger) during blood sampling. The increase of haemolysis was negligible.

	additional haemolysis (%)	**additional K^+ (mmol/l)**
"*in vivo*" haemolysis	0	
Sample 1	0.195	0.11
Sample 2	0.130	0.08
Sample 3	0.277	0.16

After the sampling step, the blood was transported into the clotting chamber, mixed with the lyophilised clotting activator thromboplastin, and allowed to clot. After clotting, the serum was extracted out of the clot via an under-pressure as already shown experimentally. Because of the pressure difference, all RBCs were stressed and some of them were lysed. Therefore, haemolysis and thus the presence of haemoglobin in the extracted serum were checked with the same method as described above. Furthermore, the remaining RBCs in the serum were counted. Table 2 summarises the quality of the sera from different experiments. There is no correlation between the haemolysis and the remaining RBCs in the serum.

Because of the high loss, the direct removal of the RBCs out of neat serum is not possible. The volume has to be enlarged by the first dilution step. Due to osmolality, dilution in pure water is not feasible. As decided at the General Assembly meeting in Oslo in October 2013, the first dilution was performed with an isotonic solution.

After the volume expansion, the filtration of the solution for the removal of the RBC can be performed.

Table 2: Summary of the serum quality after the extraction experiments. There was no correlation between measured haemolysis and the remaining RBCs in the serum.

additional haemolysis (%)	RBC count (1/µl)
1.17	22,500
2.56	168,750
1.92	625,000
0.75	1,725,000
0.37	3,450,000
1.21	28,750
0.30	90,000
1.33	165,000

FhG-ICT-IMM focused on several methods to decrease the haemolysis. One major point was the surface of the cartridge, which has to be as smooth as possible to prevent cell damage by sharp edges or spikes. This will be optimized in the final cartridge by polished injection moulding tools. Another point is the hydrophobicity of the surface. It is known from the literature, that a hydrophilic material is shielded against protein adsorption and thus the adsorption and destruction of RBCs (Desai and Hubbell, 1991). Hydrophobicity can be increased by PEG surface coating. Therefore FhG-ICT-IMM developed a simple but stable PEG coating which increased the hydrophilic properties of the surface. The first results showed that a PEG treated surface was significantly more hydrophobic. This was observable in the decrease of the contact angle from 83° to 64°.

Furthermore it is known that bovine serum albumin (BSA) works best to prevent cell and protein adsorption to surfaces. Yet it is not possible to use BSA here as albumin is measured later in the process flow. Future experiments will address this issue by using another protein, e.g. casein, thus preventing all non-specific binding.

In the near future, the sera will be compared by observing the potassium ion concentration with the ion sensor developed within the d-LIVER project in cooperation with CEA-LETI.

3.3. Liquid dilution

The standard method to dilute/mix two liquids in a microfluidic system requires two inlets and one outlet and the two liquid plugs are joined at a junction. This simple procedure effectively avoids air inclusions/bubbles, but it requires three fluidic ports and a precise fluid control.

The new concept for on-chip dilution developed at FhG-ICT-IMM is progressing constantly. Various designs were tested to optimize the dilution module and to guarantee reproducibility of the process. However, it became apparent that the designs available at FhG-ICT-IMM were unsuitable for serum, due to its wetting behaviour in plastic channels. Therefore FhG-ICT-IMM devised a new concept/design which enables the principle for liquids which also wet the cartridge material strongly. After intense development, the latest design is currently the best working for the dilution steps.

Being an essential and important part of the cartridge, the dilution chamber will be continuously improved. Therefore, FhG-ICT-IMM performed simulation-aided design optimization of the plug-burst chamber to improve its functionality and reproducibility.

In such a chamber, as shown experimentally, the first plug goes in and bursts, which means that it stays in the chamber and an air-bypass emerges. The second liquid fills the chamber completely and the entire fluid can be sucked out. FhG-ICT-IMM intends to patent this microfluidic dilution module.

3.4. Combined module for blood sampling, serum generation and dilution

Blood collection, serum generation, and dilution are the main steps in the first half of the fluidic process. After the serum generation module and the dilution module were each positively tested, both were combined in a separate microfluidic chip and tested together. The new test chip contained both modules sequentially. Firstly, the blood collection capillary was filled with 20 µl of blood. The blood plug was sucked into the clotting chamber, where the freeze-dried thromboplastin was located. The clotting started immediately the thromboplastin was dissolved. After a few minutes clotting, the serum was extracted by under-pressure from the clot. The extraction had to be performed until the serum plug reached the Teflon membrane. Secondly, a 2 µl plug was split from the serum and transported to the dilution chamber. Simultaneously, the 10 µl water plug was transported to the dilution chamber and merged with the serum plug to give a 1:6 dilution.

Ultimately, the ion content of the extracted and diluted serum will be measured by the ion sensors, which are being developed in d-LIVER.

3.5. Reagent storage

In the final microfluidic cartridge, several reagents need to be stored and handled. Some of them will be stored in a liquid form whereas others have to be dried or even freeze-dried. To realise the storage of dried reagents (thromboplastin and dried sensor surfaces) and solutions, spatial separation will be necessary. Therefore a reservoir chip for liquid storage was designed.

The reservoir has to provide the liquids listed in Table 3 for sample dilution and sensor calibration. Since separating the freeze-dried and highly hygroscopic thromboplastin and dried sensor surfaces from the lyophilized liquids is difficult if the liquids are stored on the BBC, a separate reservoir chip was designed which will be connected to the microfluidic cartridge when the blood sample enters the system.

Table 3: Liquids, necessary in the BBC.

liquid	**function**	**min. liquid volume**	**chamber volume**
ion low calibration	ion sensor calibration	5 µl	10 µl
ion high calibration	ion sensor calibration	5 µl	10 µl
isotonic solution; 0.9% NaCl	rehydration of albumin sensor surface	5µl	10 µl
isotonic solution	dilution of blood serum	10 µl	15 µl
bilirubin reference solution	bilirubin reference	5 µl	10 µl

A simple yet functional design for such a reservoir chip is depicted in Figure 6. The reservoirs containing the liquids are aligned in parallel and can be addressed individually. The connection to the microfluidic cartridge is realized with piercing inlays, which perforate membranes in the reservoir chip to establish a connection to the microfluidic network of the BBC.

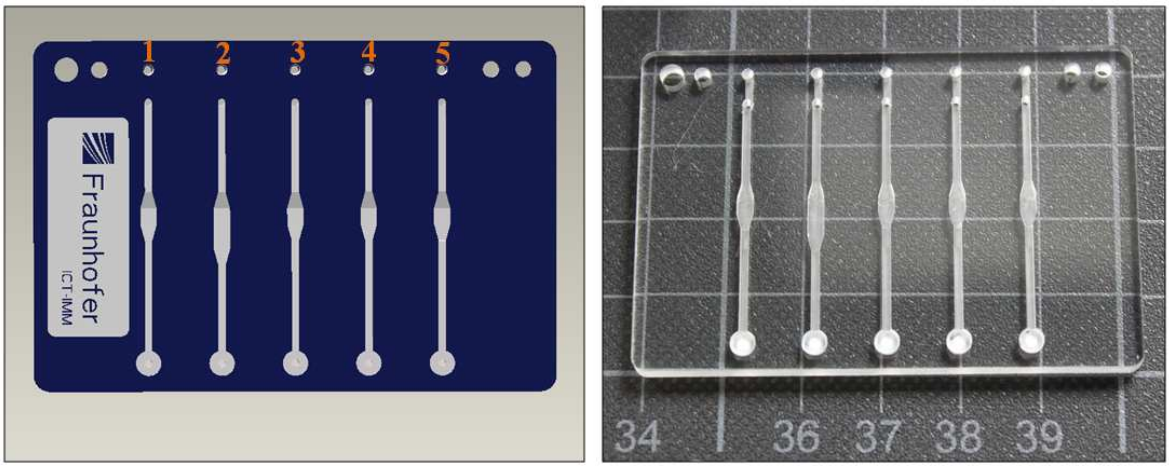

Figure 6: Preliminary design of a reservoir chip to pre-store reagents. 1) ion low calibration, 2) ion high calibration, 3) isotonic solution; 0.9% NaCl 4) isotonic solution, and 5) bilirubin reference solution.

The microfluidic cartridge and the reservoir chip can be aligned in two ways. As sketched in the left panel of Figure 7, the two chips may overlap at one end only. In this way, the cartridge can easily be connected by the BBI and all ports can be controlled from one side. The stacked design shown in the right panel of Figure 7 is more compact, much more stable mechanically, and easier to handle. However, interfacing to the cartridge is a major challenge and consequently, design A is favoured by all partners.

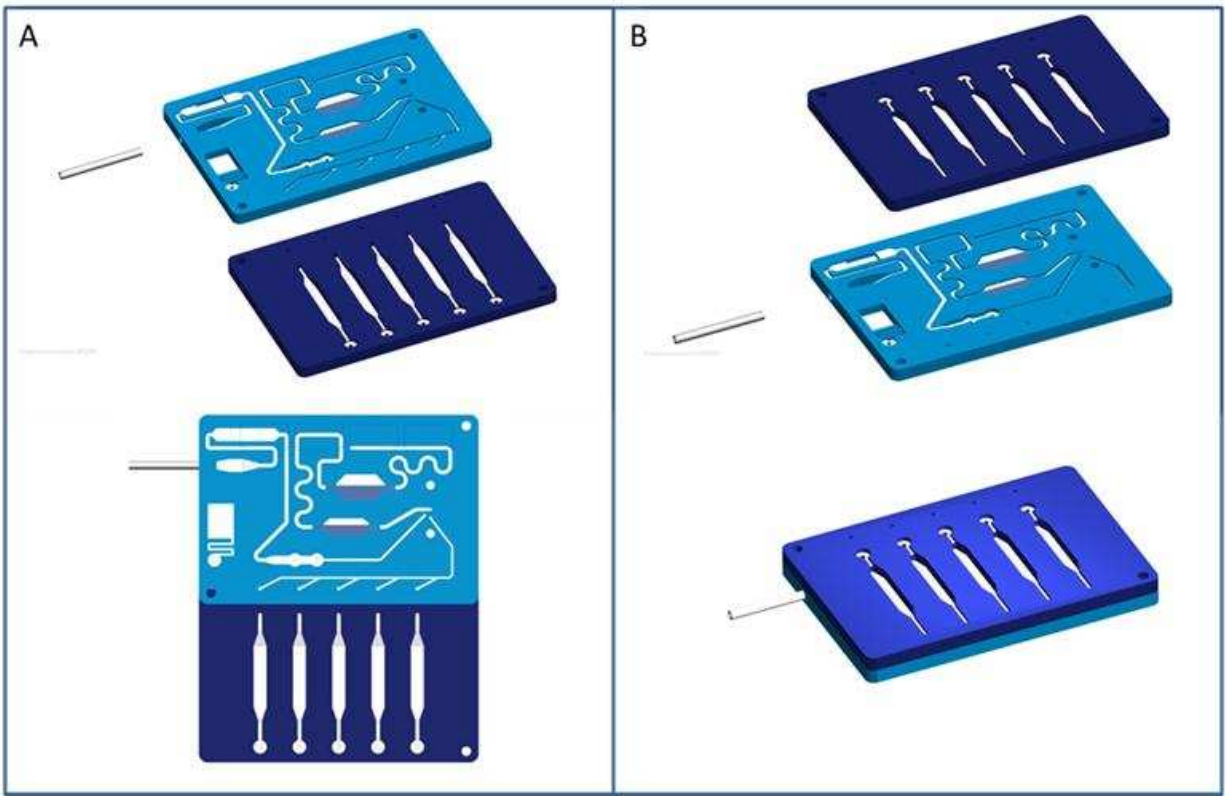

Figure 7: Possibilities to join the reservoir chip (dark blue) and the microfluidic cartridge (light blue) to the master chip. Design A is favoured.

4. Summary and conclusions

In this deliverable, the development and the function of the microfluidic modules of the blood biochemistry cartridge is reported. These microfluidic developments enable a serial fluidic pathway.

At the beginning of the microfluidic process 20 µl of blood is introduced, via the blood sampling capillary, into the microfluidic cartridge. The activated clotting takes place in the subsequent clotting chamber. The special chamber design and the optical transparency allow optical clotting measurement. Due to the necessity of potassium measurement and the fact that damaged RBCs release potassium, haemolysis was investigated. The rough surface of the micro-milled cartridges damaged the RBC. Therefore, the use of a PEG coating was tested and found to be beneficial. The required volume of serum will be extracted out of the clotted blood. After metering, the serum has to be diluted. This was realised by the so-called dilution chamber. All three modules were assembled a single test cartridge, with which the modules were tested positively in a serial workflow.

Finally, the development and optimisation of the reservoir chip for liquid storage was described.

In the end a very simple and yet effective workflow was developed and realized. All microfluidic modules perform well and contributed to the serial process. In the next step, the whole microfluidic process has to be tested. To come close to a near-final-design scenario suitable for clinical evaluation studies, the microfluidic cartridge will be realized by injection molding.

5. Literature cited

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