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MALDI-TOF-MS-based point of care device for Hepcidin analysis†

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Hepcidin, an important iron regulator in the human body which is known to be linked with Malaria, and was first discovered in urine. We investigate the design choices for a low cost microfluidic based device for the preparation of an urine sample before it can be analyzed by a Time-of-Flight mass spectrometer. Matrix Assisted Laser Desorption/Ionization has been used as a method to detect the biologically active Hepcidin-25 seperately from its other isoforms.

1 Introduction

Hepcidin (Hep) is a cysteine-rich hormone that was first discovered in urine [1] produced by hepatocytes in the liver [2]. It is a major regulator of iron in the human body. [2,3] Hep binds to the iron export transport protein ferroportin and reduces its activity. [4,5] Hep interacts with ferroportin with mostly hydrophobic interactions [6]. Urine consists of many isoforms of Hep [7] including the bioactive [8] Hepcidin-25 with 25 amino acid residues.

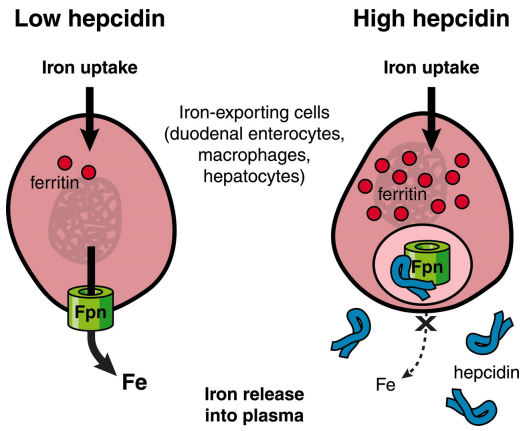

Fig. 1 Interaction of Hepcidin with ferroportin. Figure taken from [5]

1.1 Hepcidin and Malaria

Most literature points to an increased serum concentration of Hep in patients with malaria [9]. A heightened serum concentration of Hep is linked to disturbed homeostasis of iron [10] and elevated concentrations of Hep were found in the urine of patients with malarial anaemia. [11] Some studies report poor correlation of Hep and iron levels in blood, but it still correlates with regulatory and anti-inflammatory cytokines like interleukin-6 and interleukin-10 that in patients with malaria. [12] All this points to the possibility of development of a point of care device to diagnose malaria based on Hep.

1.2 Hepcidin detection methods

Current Hep detection methods can be broadly classified into Radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), ligand binding assays and Mass spectrometry. [13] RIAs have a low limit of detection, [14] specificity and linearity [15] but due to concerns about radiosafety, their use has been limited in hospitals. Reproducible, stable and sensitive ELISA assays for prohepcidin have been developed, [16] but prohepcidin levels are not consistent with iron storage or absorption parameters. [17] Other ELISA assays have been developed which have a high variation at low concentrations [18] and higher than reported limit of detection [13]. Ligand binding assays for hep [18] are not widely reported or have even been redacted. [19]

Mass spectrometery (MS) based methods such as Liquid chromatography (LC) [20] and Matrix Assisted Laser Desorption/Ionization (MALDI) [21,22] are both able to distinguish between the various isoforms of Hep. Although both methods are sensitive and fingerprint the molecules(selective), the data acquisition time for MALDI-MS is in the order of a few minutes compared to a few hours for LC-MS. [23] For this research the preferred method is MALDI-TOF-MS.

2 Theory

2.1 Sample prepration

Before a urine sample can be analysed with MALDI, it must be prepared. The protocol for preparation generally involves centrifuging, to separate larger particles and cells from urine, addition of trifluoroacetic acid (TFA), for buffering the pH, and mixing with a matrix material before transferring to the MALDI-MS analysis system. This can be achieved in a lab-on-a-chip format with microsieves [24], mixers [25] and valves [26] used in combination with electroosmotic pumping. Low concentrations of hep in urine can be detected. But, without Hep enrichment, the signal to noise ratio worsens for lower sample volumes and concentrations.

2.2 MALDI-TOF-MS

For identifying Hep-25, Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) is used. MALDI-TOF is a technique that ionizes molecules by using a laser and an energy absorbing matrix. This analysis is particularly suited for bio-molecules and organic molecules. Because of the fragile nature of these molecules MALDI is an effective method because it does not degrade the molecules.[27]

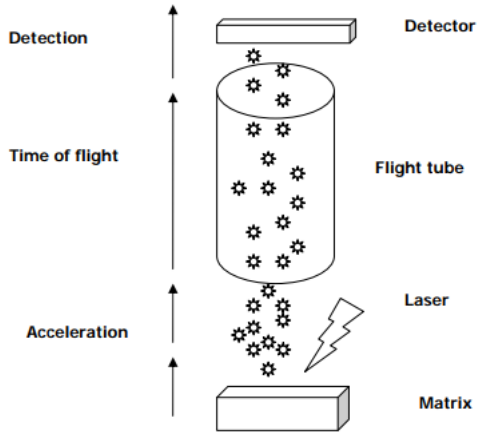

Fig. 2 Principles of MALDI-TOF technique. Figure taken from[28]

The MALDI method can be explained in a three-step process.[28] First the analyte sample is mixed with a matrix material. This matrix will be explained in more detail later. Next the sample which is crystallised in the matrix is placed on a MALDI plate. From there the sample is irradiated with a pulsed laser in the UV spectrum (10 to 400 nm)[29], which triggers ablation and desorption of the matrix and sample. The last step is that the molecules are ionized in the plume generated by the ablated gases, the ionized molecules are then accelerated in an electric field and detected by a Mass Spectrometer (MS). Figure 2 shows how this setup works from the MALDI plate to the detector of the Mass Spectrometer. Figure 3 zooms in on the irradiation process of the laser when it comes in contact with the matrix material.

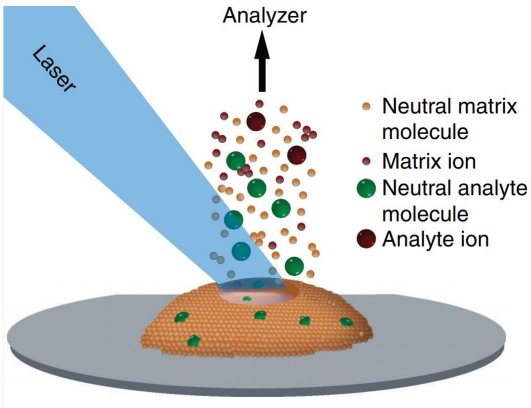

Fig. 3 Representation of irradiated matrix material and resulting plume. Figure taken from[30]

The plume coming from the matrix is accelerated in the Flight tube which is a part of the MS. This MS works with the Time-of-flight principle (TOF). The mass-to-charge ratio of ions is determined by the time that it takes for ions to reach the detector. This is possible because the distance from the matrix to the detector and the strength of the electric field are known.[31]

2.3 Matrix material

The matrix material needed for MALDI is specific to the application and sample that is being used. Optimizing the matrix material is a crucial and unique step which is different for every analysis. For a matrix material to be suitable for MALDI it should have a molecular weight that is low enough to enable easy evaporation, but high enough to not evaporate during the sample preparation steps. Matrices are often acidic which makes them suitable as a proton source which encourages ionization. Matrices also show a strong absorption for the laser source wavelength.[32] During the sample preparation the matrix material can be mixed with other materials to give it the necessary properties like viscosity, acidity and vapour pressure.

3 Experimental

Micropillar structure arrays filters[24] with interpillar spacing less than cellular dimensions can be used to replicate the effect of extracting the supernatant after centrifugation. For the identification of Hep-25, α-cyano-4-hydroxycinnamic acid (CHCA) is used as a matrix material.[33] This acid is mixed with purified water, a counter ion source of TFA[22] and the sample solution, which is urine containing Hep-25. For mixing all the different solutions active micromixers are used. These mixers work by applying an electric field induced by 4 electrodes in an oval configuration[34]. The layout of the mixer is visible in Figure 4.

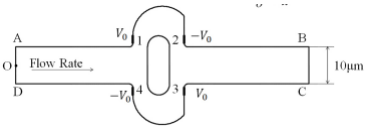

Fig. 4 Top view of an active micromixer using electroosmotic flow. Figure taken from[34]

When no Electric field is applied the different fluid streams will not mix and follow a laminar flow to the outlet of the mixer, which is visible in Figure 5. By applying the electric field the ions in the material are affected and will cause vertices in the flow pattern of the fluid. This causes the separate fluid streams to mix on a relatively short distance, which is visible in Figure 6.

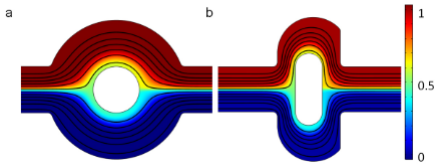

Fig. 5 Fluid steamlines when no Electric field is applied. Figure taken from[34]

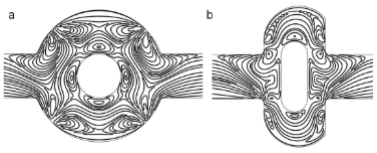

Fig. 6 Fluid streamlines under the application of an Electric field. Figure taken from [34]

Combining three of these mixers with fluid reservoirs and a sensing window for the TOF-MS results in a global layout for the lab on a chip, as shown in Figure 7.

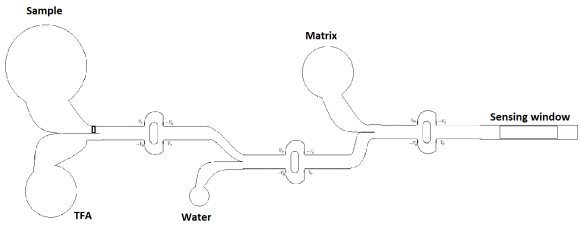

Fig. 7 Global chip layout with fluidic reservoirs for different mixtures. Picture is simplified an not to scale.

The dimensions of the entire layout is approximately 700μm x 300 μm. The dimension of the channel inside the mixer and sensing window have a width of 10μm and a height of 100 μm. Assuming urine has the viscosity and density of water, the Debye length can be calculated as follows.

$$L_D = \sqrt{\frac{\varepsilon_0 \varepsilon_r RT}{2F^2 c}} = 2.5\mu m \tag{1}$$

This Debye length is well below the characteristic dimension, the channel height. So, electroosmotic flow is feasible. The Electric field required for driving the fluid over the maximum length can be found with the following equation:

$$E = \frac{v\eta}{-\varepsilon_0 \varepsilon_r \zeta} = 125kV/m \tag{2}$$

Applying this field along the length of the chip would require a voltage of 88 V, which is feasible in a handheld device.

3.1 Fabrication

For bed side care, the chip needs to be low cost and single-use. The favoured material for satisfying these requirements is a polymer. Hep is an amphiphilic protein [35], which means that it contains both hydrophobic and hydrophilic residues. Therefore Polytetrafluoroethylene (PTFE) is chosen as the material for the substrate. PTFE is an omniphobic, non wetting polymer which means that generally nothing sticks to it. Some problems arise with the bonding of PTFE because of its omniphobicity. This will be discussed later. On the bottom PTFE substrate the microchannels and other fluidic manipulating structures are formed using hot embossing [36]. This method is preferred because some of the structures, like the mixers, are too complex to be fabricated with injection moulding. For the hot embossing a master mould is created using the standard lithography and etching. After fabrication a second substrate of PTFE is placed on top of the first substrate to seal the fluidic channels.

Before this happens several additional steps are performed on both substrates. First the MALDI plate (metal) and gold electrodes are sputtered at the required positions. The electrodes are sputtered around the mixers and at every reservoir. The MALDI plate is sputtered in the detection window. The MALDI plate is necessary to improve the process of irradiation of the matrix material for the Mass Spectrometry. With the microchannels fabricated and metals sputtered, valves can be created to keep the solutions inside the reservoirs until the correct time. This needs to be a valve that is normally closed, the valve can be opened with the pressure the liquid is exerting on a movable membrane. Inside the channel a small block of PTFE was left standing during the hot embossing step. On top of that an easily movable membrane can be placed, in this case a thin membrane of TPU [26]. This membrane is fixed on the top substrate using a photo-curable adhesive [37]. The top substrate has a hole etched where the membrane has room to move. This structure is visible in Figure 8, the PTFE film and layer can be combined to a single layer. Where it says PMMA this is PTFE for this research. If pressure is applied on top of the reservoir the force the liquid exerts on the membrane increases. At a certain pressure the membrane will displace and the liquid will be allowed to move through. [26] In Figure 9 it is visible how the side-view looks.

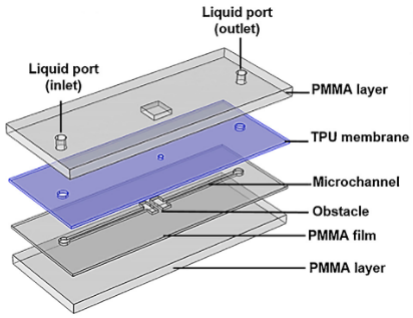

Fig. 8 Exploded view of the different layers. PMMA should be seen as PTFE. Figure taken from [26]

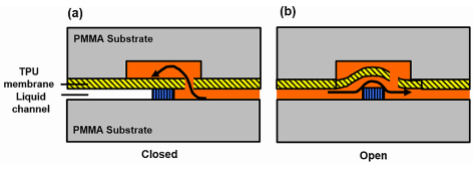

Fig. 9 Working principle of micro check-valve in the channel. Taken from [26]

With the valves in place the reservoirs are filled with their respective solutions. After that the chip is sealed with the second PTFE substrate. As we already know the top substrate has a hole for the TPU film to be able to move. Also, a hole is etched for the detection window. It is important that this detection window

is open because the plume of ions needs to be able to move towards the detector of the Mass Spectrometer. The substrates are fixed with thermal fusion bonding. After the bonding the fluidic accesses and electrode accesses are drilled in the top substrate. Above the reservoirs holes are drilled to access the TPU thin film on which a pressure can be applied on the liquid. This application of the pressure will be done with the device in which the chip is inserted to connect to the power supply and perform the MALDI-TOF-MS.The result is a chip with a sealed channel, three already filled reservoirs, a fluidic access for the sample and an open detection window for the TOF-MS.

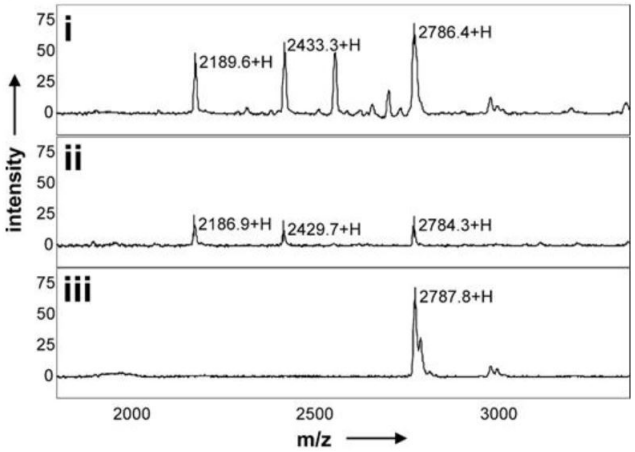

Fig. 10 Mass spectrum for Hepcidin-25 in urine of (i) patients with iron disorders (ii) in urine of a healthy volunteer (iii) Calibration solution containing synthetic Hepcidin-25 [38]

4 Results & Discussion

The fabricated chip is able to do the sample preparation and mixing within a time of 17 minutes. This is a lot faster compared to conventional MALDI sample preparation which can take up to several hours and requires skilled technicians and a lot of intermediate steps. This preparation time is based on the flow rate to move $5\mu L$ of solution to the detection window.

$$Q = v \cdot A = 5 \cdot 10^{-4} \mu L/s \tag{3}$$

$$\frac{5\mu L}{Q} \approx 16.7 minutes \tag{4}$$

The Mass spectrometry separates the ions based on their mass to charge ratio, thus unique peaks are obtained for each and every ion from the matrix-sample mixture. This is done by irradiating the matrix solution, which is located on the detection window, with a laser. This laser is positioned in the TOF-MS in which the chip is placed. The result of the TOF-MS is a Mass spectrum. The mass spectrum of a measurement is visible in Figure 10. The area under the charge to mass ratio vs intensity curve (Mass spectrum), defines the amount of substance present in the sample. A second measurement with an artificially spiked Hep-25 concentration can be used as a calibration measurement. By comparing the mass to charge ratio the correct peaks can be matched and the concentration of Hep-25 is found. In Figure 10 (i) we see three major peaks corresponding to Hepcidin-20 Hepcidin 22 and Hepcidin-25 (Left to Right). The concentration of Hep in a healthy volunteer Figure (10 (ii)) is lower than in patients with iron disorders. The calibration internal standard solution containing only Hepcidin-25 only has one major peak corresponding to it, as is visible in Figure 10 (iii) .

Looking at the figure we see a clear similarity in peaks between the calibration measurement and the two measurements of urine samples.

5 Conclusion & Outlook

It is possible to fabricate a lab on a chip which can perform the Hep-25 analysis based on MALDI-TOF-MS. The concentration of the bioactive isoform Hepcidin-25 can be found independent of other isoforms of Hep.

The sample preparation can be performed in approximately 17 minutes which is a lot faster compared to conventional sample preparation for MALDI-TOF-MS. The process of the actual TOF-MS is performed by a device in which the chip is inserted. This device provides the electrical power to create the Electrical field, is able to put pressure on the reservoirs to open the fluidic valves and can perform the actual Mass Spectrometry by irradiating the sample with a laser and analysing the mass/charge ratio based on the Time-of-Flight of the ions. The total measurement is approximated to take 30 minutes.

The results show that a clear relation is visible between a calibration measurement and measurements from patients with raised Hep-25 concentrations. Even normal Hep-25 concentrations can still be distinguished with the MALDI-TOF-MS method.

A recommendation for further research would be to further investigate the low concentration of Hep. A method to improve the concentration would by the dried droplet method. The downside of this method is that it complicates the system and increases the total time of a measurement. When downscaling to lower sample volumes the signal to noise ratio should be taken into account to make sure there are still measurable results.

Another critical point is that in this research the focus was not on the machine that performs the actual Mass Spectrometry. Therefore it is not entirely known if such a machine would be a handheld device to enable bedside Hep-25 analysis. The fabrication of this lab-on-a-chip can push the industry towards working on handheld devices to improve bedside analysis.

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