MICROFLUIDIC LAB-ON-A-CHIP SYSTEM WITH INTEGRATED SAMPLE PREPARATION FOR PROCESSING IMMUNOASSAYS

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ABSTRACT
We present an advanced, injection molded microfluidic lab-on-a-chip cartridge to process immunoassays with unit operations for sample preparation, metering, mixing, incubation, washing, chemiluminescence detection and waste handling. A newly developed readout device is capable of recording data points under rotation. Latest assay results feature a lower average standard deviation (7 % of maximum signal) in contrast to previously achieved 27 % [1]. A competitive chemiluminescent Estradiol immunoassay is demonstrated on-chip with a series of different Estradiol concentrations. A lower limit of detection of 60 pg/mL is established with a time-to-result of 45 min compared to 60 min in a microtiterplate.

KEYWORDS: Immunoassay, Centrifugal Microfluidics, Chemiluminescence

INTRODUCTION
The liquid handling of heterogeneous immunoassays requires sequences of adding and removing reagents from and into a test environment in which antibodies interact with an analyte. The most common representative hereby is the solid phase immunoassay. Capture antibodies are bound to a substrate and subsequently exposed to a sample and a labeled detection element. As a result the concentration of potential target antigens correlate to a signal generated by the labels in combination with a suitable substrate. The requirement for lab-on-a-chip systems is therefore to provide a flexible platform with microfluidic unit operations enabling sequential reagent processing. There are numerous approaches realizing a microfluidic lab-on-a-chip platform or micro total analysis system, for example pressure driven systems [2]. These systems however require additional external pumps and connections that can add to the complexity of the complete system, making it difficult to handle in a point of care environment. Another approach is to implement diagnostic tests on centrifugal microfluidic platforms. These systems utilize centrifugal and capillary forces to route reagents between so called unit operations. These unit operations represent assay steps interpreted in microfluidic structures on the chip.

FUNCTIONAL PRINCIPLE
Figure 1 shows the fluidic layout and the single assay steps processed by the interconnected unit operations. The assay which is used for demonstration is a competitive Estradiol chemiluminescent immunoassay (CLIA). Antigens from a sample and competitor antigens conjugated with horseradish peroxidase (HRP) are mixed and exposed to a solid phase with immobilized capture antibodies. More antigens present in the sample lead to statistically less competitor antigens that bind to the capture antibodies. With a decreasing number of labeled competitor antigens bound to the capture antibodies the chemiluminescent signal also decreases. Therefore this kind of assay is called competitive immunoassay. One of the main challenges of realizing a solid phase immunoassay on chip is to immobilize capture antibodies on the substrate. The main philosophy is to create a flexible platform for different kinds of assays. Our approach is to use goat-anti-rabbit antibodies as primary coating of the reaction chamber. This technique offers the possibility to bind any antibodies produced in rabbits on top as the actual assay capture antibody.

Each microfluidic cartridge fabricated of polystyrene accommodates 4 test structures. Each of these structures can be used to perform one test. Six cartridges can be mounted on the rotor of the readout device allowing to process 24 tests at the same time. A hydrophilic coating with polyethylene glycol (PEG) enhances capillary priming of siphons while an additional local coating with Teflon Carbon Black is used to create hydrophobic barriers where required (see Figure 1). Three inlets are used for injection of all necessary reagents.

The readout device accommodates a photoncounter module (Hamamatsu H7467) connected to a lightguide which is fixed beneath the radial position of the reaction chambers. A total number of 3600 data points can be recorded per rotation. The readout frequency is set to 8.3 Hz. Multiple turns can be recorded resulting in integrated signals emitted at the predefined azimuthal positions of the 24 reaction chambers. For recording a calibration curve, standard samples were spiked with different concentrations of Estradiol and processed on-disc.
Figure 1: A) Schematic layout of one immunoassay structure. B) Schematic illustration of the fluidic processes of a competitive assay: 1) Whole blood is dispensed into the blood plasma separation and metered. 2) Accelerating to 50 Hz separates the plasma from the blood cells. The extracted plasma is routed into the mixing chamber together with the competitor antigens from inlet 2 resulting in a total volume of 60 µL. 3) An unidirectional shakemod [3] is used to create a homogeneous mixture. 4) By decelerating to 0 Hz, the capillary siphon primes. Accelerating to 6 Hz empties the mixing chamber and the fluid is routed into the reaction chamber. 5) Potential antigens and competitor antigens bind to immobilized antibodies during incubation. 6) After incubation, acceleration to 12 Hz causes the fluid to overcome the inline hydrophobic barrier coated with Teflon Carbon Black followed by a 0 Hz step allowing the siphon to finish priming. Washing buffer is dispensed into inlet 3. The disc is accelerated to 12 Hz which causes the reaction chamber to empty into the waste reservoir due to the additional volume. 7) Three washing steps are performed. Each washing step requires 60 µL of washing buffer that are first spun into the reaction chamber followed by an additional volume of 60 µL, emptying the reaction chamber into the waste reservoir. 8) For chemiluminescent signal generation, 60 µL of a substrate solution of H2O2 and luminol is dispensed into inlet 3 and spun into the reaction chamber.

Figure 2: Estradiol assay protocol: 1) Antigens and competitor antigens are mixed in the mixing chamber. 2) The resulting mixture is routed into the reaction chamber and left for incubation (30 min). 3) After washing, the substrate is added, causing a chemiluminescent signal in combination with horseradish peroxidase.
EXPERIMENTAL

In order to demonstrate the liquid routing steps, a protocol for Estradiol detection from standard solutions was established. The validation of the system with whole blood is pending. A mixture of antigens and competitor antigens were directly pipetted into inlet 2 and spun into the mixing chamber. A fluidic protocol starting from step 4 (see Figure 1b) was processed. Six Cartridges were processed on two consecutive days resulting in 4 data points per Estradiol concentration. The yield of the microfluidic network depends mostly on the reliability of the siphons and therefore the coatings. Since all coatings were applied manually (PEG, Teflon Carbon Black) this preparation step and the antibody coating are the most critical parts while preparing the cartridge for the assay.

RESULTS AND DISCUSSION

 Estradiol concentrations of 0, 25, 100, 250, 500 and 1000 pg/mL were tested. A significant reduction concerning standard deviations (4 times lower) in contrast to previous results [1] was achieved due to the newly developed readout device and an improved chip coating protocol. A yield of 95 % was evaluated for the microfluidic network functionality, where malfunctions are likely caused by manual production steps as mentioned above. A lower limit of detection of 60 pg/mL (threelfold standard deviation of the negative control) was established with a time-to-result of 45 min compared to 60 min in a microtiterplate.

![Figure 3: A) Compiled Estradiol assay results of 6 cartridges processed on two consecutive days. Six concentrations were tested. Each data point comprises 4 incubation chambers. Points are fit by an exponential decay. B) Comparison between on chip test and microtiterplate reference.](image)

CONCLUSION

A microfluidic concept for immunoassay processing is realized. Latest assay results show the successful implementation of an Estradiol assay. The versatile microfluidic layout allows for performing different kinds of assays. For example sandwich assays can also be processed utilizing the cartridge and the new readout device. This also creates the possibility to establish panels of immunoassays where a combination of two different assays corresponds to a clinical diagnosis. Future work will involve automation of cartridge fabrication (coatings), alternate antibody immobilization and assays with whole blood.

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