LIPOPHILIC-MEMBRANE BASED ROUTING FOR CENTRIFUGAL AUTOMATION OF HETEROGENEOUS IMMUNOASSAYS

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ABSTRACT

We demonstrate centrifugal [liquid handling] automation of an Enzyme-Linked Immuno-Sorbent Assay (ELISA) for the detection of anti-p53 antibodies in whole blood. On this "Lab-on-a-Disc" (LoaD) platform, all unit operations were implemented by event-triggered rotational flow control. In order to avoid interference during absorbance measurement from the solid phase in this heterogeneous assay format, it is pivotal that the intermediate reaction product is eventually forwarded from the incubation chamber to a distinct optical measurement chamber. To this end we have devised routing of flows by lipophilic film valves (LFVs) which remain intact in aqueous and selectively dissolve when exposed to an ancillary, oleophilic solvent.

INTRODUCTION

In this work we specifically consider centrifugal microfluidic systems towards the development of conceptually simple and cost-efficient bioanalytical point-of-care (POC) applications [1]. The full integration and automation of underlying liquid handling protocols while keeping the instrumentation compact and cost-efficient is a common design goal of these "Lab-on-a-Disc" (LoaD) systems [2]. As during spinning all liquids are subject to the same radial force field, valves have to be opened selectively in time and space for implementing sequential and parallel process steps. Many early-stage LoaD systems have introduced capillary barriers or siphons to orchestrate flow control. Recent developments showed the integration of functional materials into microfluidics [3], for instance disc-based ferrowax plugs were molten by an external laser [4], thus increasing the complexity of the instrument. Also centrifugo-pneumatic dissolvable films (DF) valves were presented which are opened by rotationally induced contact with aqueous solutions such as most biosamples and reagents [5].

We introduce here a novel, sacrificial membrane which is dissolvable in lipophilic liquids. This flow control element initially blocks aqueous solutions until an ancillary, oleophilic liquid is introduced from its back side to route the next aqueous solution, e.g. an elution buffer, to designated outlet. This event-triggered [5] router is implemented to demonstrate the on-disc integration of a bead-based, i.e. heterogeneous immunoassay.

In comparison to planar surfaces in bioassays, microparticle based assays provide much higher surface area thus substantially improving performance and have hence found direct applications in various areas like immunoassay formats (e.g.: ELISA) and bioprocessing steps like purification and extraction [6]. Typically such applications feature several steps of washing and incubation. Bead-based ELISAs are an essential tool for the detection of antigens and antibodies for clinical diagnostics. The final step in such assays is commonly the reaction of the enzymatic substrate in the bead column (immobilized capture antibodies on bead surface in the reaction chamber) and then eventual quenching with an acid before measuring the absorbance values. In an automated point-of-care ELISA, the absorbance measurement is ideally performed on disc, thus requiring a clear pathway for optical detection. The presence of a solid phase hence makes it mandatory to route the eventual enzymatic substrate away from the reaction chamber to a final quenching / measurement chamber to avoid any disturbance in absorbance measurements of the end product.

In LoaD systems, this has been accomplished previously by using laser-actuated valves [4]. We address this issue by introducing a routing mechanism based on lipophilic membranes that are selectively dissolved on demand by an immiscible liquid. This "transistor mode" operation with an ancillary liquid makes them particularly interesting since the routing does not require any additional instrumentation other than the simple spindle motor while allowing purely rotational flow control over the fluid flow.

Using lipophilic membrane routing, we present here the integration of a multi-step ELISA assay encompassing plasma separation from whole blood, incubation with capture antibodies on beads, multiple buffer washes and eventual routing of post-reaction enzymatic substrate to a measurement chamber performed on the disc in its entirety based on event-triggered rotational flow control [5].

SYSTEM DESIGN

Lipophilic valve based routing

Lipophilic membranes are biocompatible materials [7] that are hydrophobic in nature and selectively dissolve in bio-reagent grade oleophilic solvents such as light oil [8]. When in contact with an aqueous solutions, the hydrophobic nature of the membrane keeps the liquid from wetting and dissolving it. On the contrary, oleophilic solvents wet and dissolve the membranes while being immiscible with aqueous solutions. This implies that the membranes can act as a solvent-selective, intermediate barrier between two channels. Additionally, due to their softness and plasticity, they can be easily assembled into multi-layer valve composites cut into shapes.

We have devised a 3D structure to integrate such membranes into our microfluidic flow system (Fig.1). The lipophilic membrane is integrated under a microchannel in order to restrict the flow of aqueous solutions, acting as a transient barrier for fluid communication between two

separate channels. When an aqueous solution is required to be routed from the top channel away from the normal exit, the lipophilic membrane is dissolved by mineral oil to open up the second channel and direct the subsequent aqueous solution into a designated chamber. (Fig.2)

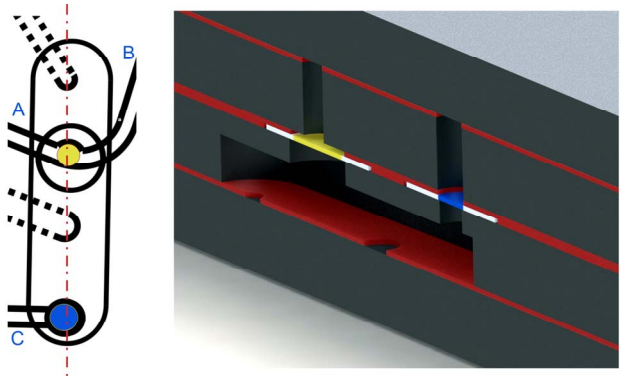

Figure 1: Lipophilic membrane based router. Cross section shows the placement of the valves inside the router. (Yellow tab- Lipophilic membrane, Blue tab- DF, Red layers-PSA; Black layers- PMMA). When the lipophilic valve is triggered, the aqueous solution from the inlet channel A routes to channel C instead of the usual channel B.

Figure 3 shows a fully integrated system for a disc-based ELISA for the detection of anti-p53 antibodies in whole blood. Individual components are: A) plasma extraction, B) reagent storage, C) bead incubation with surface-immobilized p53 capture antibody, D) waste, E) pneumatic chamber and F) the solvent-selective router. The vector of dissolvable-film valves in the waste (D) orchestrates the serial release of wash and ELISA reagents to the incubation chamber (C) as well as the ancillary liquid originally stored in chamber F to the back of the lipophilic membrane. Mixing in (C) is boosted by its centrifugo-pneumatic coupling to the compression chamber E while swiftly varying the spin rate. The incubation chamber (C) features two outlets, one of them leading via a siphon to the waste (D). The second outlet is initially sealed by a lipophilic membrane and opened to the detection chamber G by introducing the oleophilic ancillary liquid (F) from the reverse side.

In the first steps of the ELISA, sample, wash and reagents are routed from their respective reservoirs (A, B1-4) through the incubation chamber (C) where they are rotationally stirred and subsequently guided through the siphon into the waste (D). The final trigger concurrently releases the colour-generating enzymatic substrate (TMB) into C as well as the ancillary liquid to wet the first lipophilic valve. The concurrent dissolution from the backside of the second lipophilic membrane in the routing chamber with the ancillary liquid then routes the TMB from the reaction chamber through the centrifugally preferred outlet to G where the preloaded acid quenches the reaction before absorbance detection (Fig.2).

METHODOLOGY

Disc fabrication and assembly

The LoaD is an eight layered structure composed of four layers of poly methyl-methacrylate (PMMA, 1.5 mm thick) bonded by intermediate pressure sensitive adhesive sheets (PSA, 86 µm) as shown in Fig.4. All features on the PMMA layers (Disc OD 130 mm, vents, reservoirs, router components) were laser ablated (30-W CO_2 laser, Epilog, USA). The PSA layers were structured with a knife cutter (Graphtec, Japan). The topmost PSA layer consists of the bulk of the microchannels connecting the reservoirs containing the sequence of unit operations of the ELISA. Appropriate air exits are engraved in the PMMA layer above this in the top Vents layer.

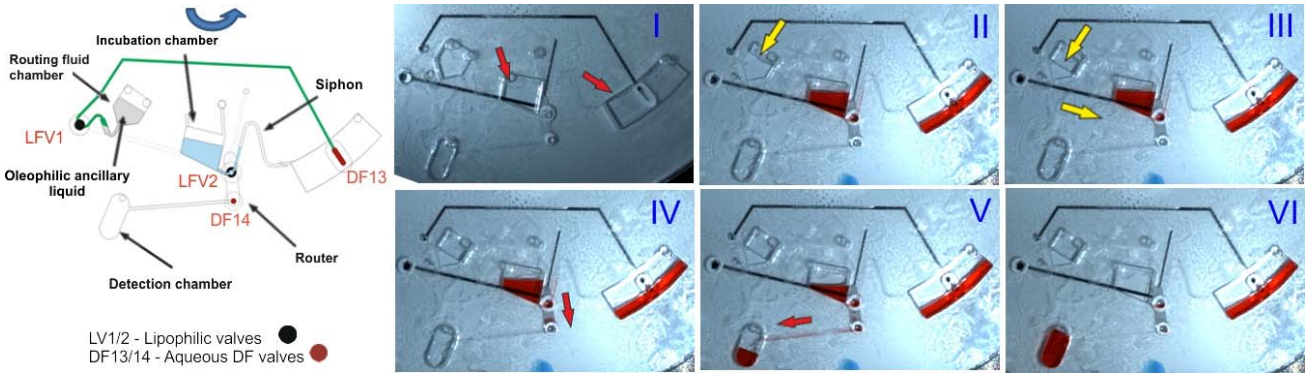

Figure 2: Trigger and release mechanism for the lipophilic-film valves (LFV) for routing of aqueous solutions. The aim is to route the final liquid from the incubation chamber (containing beads) to the detection chamber rather than across the siphon to waste. Red and yellow arrows indicate flow of aqueous and oleophilic ancillary liquid, respectively. Please note that steps II through IV are performed at spin frequency higher than the critical frequency of the siphon. (I) Aqueous solution from incubation chamber flows over to waste through the siphon triggering the release of the ancillary liquid in the routing fluid chamber. (II) DF13 valve triggers the ancillary liquid to wet the LFV1. (III) Once LFV1 is dissolved, the oleophilic liquid flows to the Router (engraved in a lower layer) and wets LFV2 but not the DF14. (IV) LFV2 is dissolved in the ancillary liquid releasing the aqueous solution from the incubation into the routing chamber where it phase separates below because of higher density enabled by centrifugally induced stratification of aqueous and oleophilic phases. (V) The aqueous solution wets DF14 thus opening up the channel to the detection chamber as in Section VI.

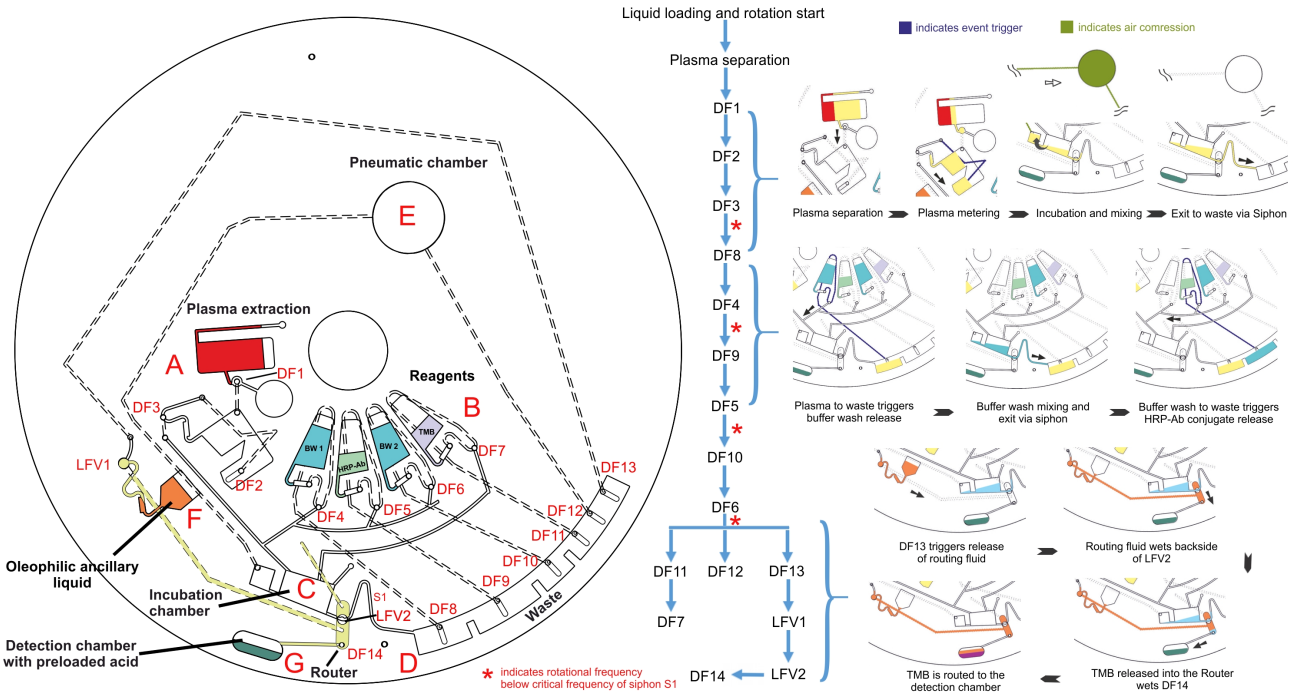

Figure 3: ELISA miniaturization on a centrifugal microfluidic Lab-on-a-Disc (LoaD) platform with event flow. The LoaD integrates all assay steps ranging from plasma extraction to the eventual absorbance measurement for quantification. The disc architecture is constituted by chambers for: A) plasma extraction, B) reagent storage (BW-buffer wash, HRP-Ab – bioconjugate of HRP with secondary antibody, TMB- substrate), C) incubation, D) waste, F) router (highlighted in yellow are the elements of the lipophilic routing mechanism) and G) detection chamber. Dissolvable-film valves (DFs) in the segments of the waste chamber (D) event-trigger the successive release of liquids along the ELISA. The final dissolution of the lipophilic film valve (LFV2) at the other outlet of (C), routes the final TMB solution to detection chamber (Fig.2).

The reservoir PMMA layer includes storage, incubation chambers and waste chambers. The DF and lipophilic membrane valves are sandwiched in between the DF support and DF cover PSA layers. The lower air channels PSA layer includes the pneumatic connections that are the core of the event-triggered structure allowing the release of reagents, buffer washes and the routing fluid on the disc. The lowest Base PMMA layer contains the vent for the router and seals the lower channels. All microchannels are 500 µm wide except for the oleophilic liquid (which measures 800 µm). All features of the device were designed using SolidWorks 2013 (Dassault systems, USA). Routing of the fluids using DF valves and event-triggered formation was accomplished as demonstrated before [5].

Protocol for anti-p53 antibody ELISA

A 140-µl blood sample is centrifuged for 5 minutes in the separation chamber to extract a plasma volume of 40 µl. This metered volume is incubated with the preloaded capture antibody + p53 protein coated beads in the incubation chamber for 10 minutes. During these steps, the mixing is induced by pulsed compression / decompression of a pneumatic chamber connected to the main incubation chamber (Fig. 3). Thereafter the beads are washed with the first buffer wash of 80 µl for 30 seconds. This is followed by incubation of the beads with the HRP tagged Secondary Antibody (40 µl) for 10 minutes. Then the final buffer wash (80 µl) is released and it incubates with the beads for 30 seconds. The last trigger after this wash eventually releases the enzymatic substrate TMB (40 µl) and also the ancillary oleophilic liquid. The TMB incubates for 7 minutes with the beads to undergo color change to blue. The LFV router sets the point in time for the release of the TMB from the incubation chamber to the final detection chamber. The reaction is then quenched by the pre-loaded 40 µl of acid in the final measurement chamber. The resulting solution is measured for absorbance at 425 nm.

Figure 4: 3D render of the exploded view of the eight layered ELISA disc showing transparent PMMA layers with intermediate PSA layers (red) for bonding. In descending order from the top: 1. Vents, 2. Micro-channel, 3. Reservoirs, 4. DF Cover, 5. DF support, 6. Valving routes layer, 7. Lower air channels, 8. Base with vents.

RESULTS

Microfluidic testing

A fully operational microfluidic routing of a fluid using a lipophilic membrane is demonstrated in Fig. 2. The purpose is to route an aqueous fluid from the main incubation chamber to the detection chamber as opposed to the normal route that is taken by the aqueous solution across the siphon. In a multi-step operation the last liquid to enter the waste event triggers the release of the oleophilic solution into the router. The sequential dissolution of the two lipophilic membranes in the router arrangement (LFV1 and LFV2) by the ancillary liquid eventually triggers the release of the aqueous solution from the incubation chamber into the router. Here the wetting of the DF14 valve by the aqueous solution (assisted by the centrifugal stratification due to lower density ancillary liquid) opens up the channel leading to the detection chamber.

Effect of reduced reagent/buffer volumes on ELISA sensitivity

One of the most important aspects of moving to a microfluidic platform from the standard bench-top ELISA protocol is the significant reduction in the volumes of the reagents and the buffer washes. For on-disc implementation, standard volumes used on 96 well plates (100 µl) were reduced to 40 µl for the TMB and Sec-Ab conjugate, and two single intermediate 80 µl for the buffer washes (instead of two 100 µl 3X washes i.e. 300 µl each wash). Figure 5 summarizes the results for the detection of the anti-p53 antibody on a well plate using the reduced on-disc volumes; the limit of detection (LOD) was determined as 0.7 ng ml^{-1}. In comparison to data from bench-top protocol, we found no significant reduction in the sensitivity of the ELISA (data not shown).

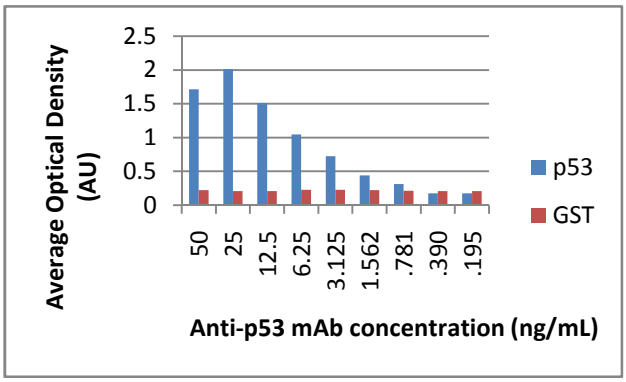

Figure 5: Detection of anti-p53 antibodies using ELISA with on-disc 40-µl volume (2.5 times lower than the bench top protocol) for reagents and a single 80-µl wash buffer. Average optical density of WT p53 proteins probed with monoclonal anti-p53 antibodies and detected using HRP secondary IgG is plotted against concentration (realizing an LOD of 0.7 ng ml^{-1}.)

DISCUSSION AND CONCLUSION

We have integrated selectively dissolvable lipophilic membranes into a centrifugal microfluidic platform for routing of aqueous solutions without the need for external actuation modules on the instrument other than a simple, frequency-controlled spindle motor. We have shown its direct applicability for bead-based ELISAs where further downstream sample handling is required before eventual detection. This is a significant improvement on current systems that require complex instrumentation approach for such routing. We also have demonstrated a fully functional microfluidic system for a bead-based-ELISA to detect anti-p53 antibodies in blood.

Further steps will include the optimization of the on-disc protocol of the ELISA. We also plan to extend the platform to further clinically relevant antibodies towards a flexible point-of-care device. Such a lab-on-a-disc system holds significant potential where intensive instrumentation and sample preparation are least desired.

ACKNOWLEDGEMENTS

This work was supported by the Science Foundation Ireland under TIDA grant Nos. 13/TIDA/B2692 and 10/CE/B1821, and the ASU-DCU Catalyst Fund.

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