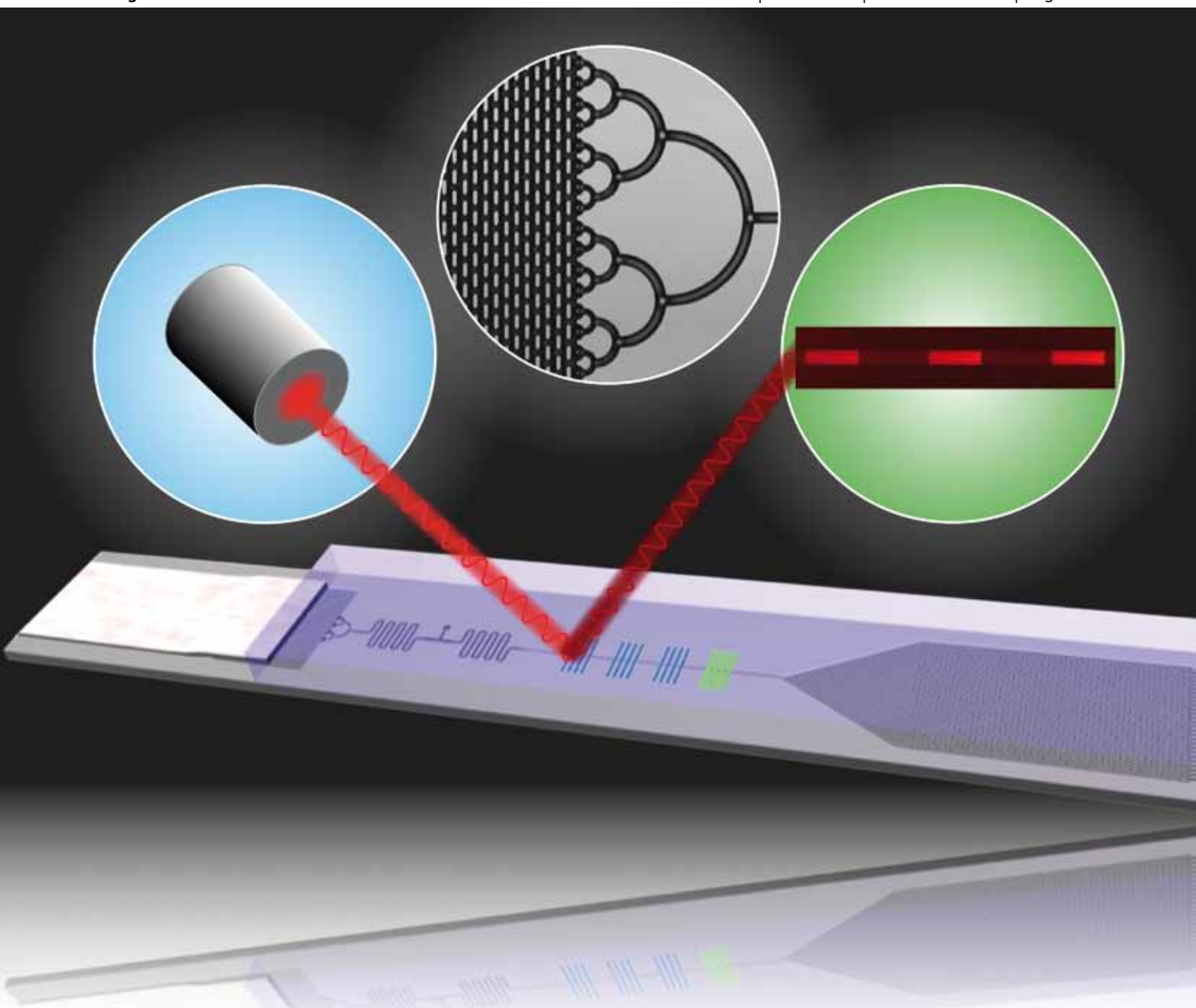


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Delamarche
One-step immunodiagnostics

Lu
Pressure measurement

Lee
Label-free detection with SERS

Demirci
Automated cell quantification for HIV



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Toward one-step point-of-care immunodiagnostics using capillary-driven microfluidics and PDMS substrates†

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Point-of-care diagnostics will strongly benefit from miniaturization based on microfluidics because microfluidics integrate functions that can together preserve valuable samples and reagents, increase sensitivity of a test, and accelerate mass transport limited reactions. But a main challenge is to incorporate reagents into microfluidics and to make microfluidics simple to use. Here, we integrate microfluidic functional elements, some of which were developed earlier, and reagents such as detection antibodies (dAbs), capture antibodies (cAbs) and analyte molecules for making one-step immunoassays: the integrated device only requires the addition of sample to trigger a cascade of events powered by capillary forces for effecting a sandwich immunoassay that is read using a fluorescence microscope. The microfluidic elements comprise a sample collector, delay valves, flow resistors, a deposition zone for dAbs, a reaction chamber sealed with a polydimethylsiloxane (PDMS) substrate, and a capillary pump and vents. Parameters for depositing 3.6 nL of a solution of dAb on the chip using an inkjet are optimized and the PDMS substrate is patterned with analytes, which provide a positive control, and cAbs. Various storage conditions of the patterned PDMS are investigated for up to 6 months revealing that storage with a desiccant preserved at least 51% of the activity of the cAbs. C-reactive protein (CRP), a general inflammation and cardiac marker, is detected using this one-step chip using only 5 μL of human serum by measuring fluorescent signals from $30 \times 100 \mu\text{m}^2$ areas of the PDMS substrate in the wet reaction chamber. The one-step chip can detect CRP at a concentration of 10 ng mL^{-1} in less than 3 min and below 1 ng mL^{-1} within 14 min. The work presented here may spur the adoption of fluorescence immunoassays using capillary driven microfluidics and PDMS substrates for point-of-care diagnostics.

Introduction

Point-of-care diagnostics are tests administered directly at the side of the patient. Small sample volumes can be analyzed and results are obtained rapidly. Devices used for point-of-care diagnostics are portable, low cost and easy to use. They can be used by health care professionals in hospitals as an alternative or complement to clinical analysers. Medical staff and patients can also use point-of-care diagnostics outside of the hospital, in the field, and in the developing world.^{1,2} They are often called one-step assays, or one-handling step assays, a name which refers to the fact that results can be obtained simply after the introduction of sample onto the device.

Point-of care diagnostics are versatile. They can be used in a variety of situations such as to measure disease markers,^{3–7} to monitor therapies,⁸ and to detect chemical and biological hazards.⁹ Point-of-care diagnostics are especially useful at

assessing health conditions that can rapidly occur and worsen. For example, the rapid diagnosis of heart problems is crucial to administer the appropriate treatment and to increase the chances of survival of the patient. In this case, the measure of the concentration of cardiac markers in blood is used to assess the cardiac risk profile of patients, the presence of myocardial injury and to predict the survival rate after a myocardial infarction.^{10,11} Monitoring the evolution of the disease requires the analysis of cardiac markers to be performed periodically. For these reasons, there is a need for fast and accurate point-of-care devices for detecting cardiac markers and more generally for detecting markers relevant to diseases that can evolve rapidly and in varying circumstances.

Many of the available point-of-care devices on the market are based on immunoassays. Immunoassays are a common technique used to detect antigens. Immunoassays for diagnostics are available in a variety of different formats ranging from the large high throughput clinical analysers confined to central laboratories to portable single use lateral flow tests. Clinical analysers measure the concentration of antigens and can measure several antigens sequentially. They require large sample volumes of a few hundred microlitres and provide a time to result of up to an hour. The simplest and most commercialized point-of-care tests on the market follow the principles of lateral flow assays in which an antigen is detected to be above a certain threshold. Lateral flow tests are low cost, portable and require a few tens of microlitres

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† Electronic supplementary information (ESI) available: The supplementary video shows a one-step immunoassay occurring on the surface of the PDMS in the reaction chamber. The video shows the flow of dAbs in the reaction chamber and the evolution of the signal after human serum spiked with CRP at a concentration of 1 ng mL^{-1} was added to the chip. The video is 16 min 30 s and is accelerated here by 40 times. See DOI: 10.1039/b906523g

of sample. One such lateral flow test that has become ubiquitous is the pregnancy test.¹²

There are several examples of successful point-of-care diagnostics devices found on the market. It is common to employ an external reader for measuring and recording the signal when accurate tests are sought. The i-STAT®¹³ is a portable analyser that uses disposable cartridges. Each cartridge performs an immunoassay that quantitatively detects one cardiac marker. The Biosite® Triage®¹⁴ is capable of multiplexed quantitative detection of 3 cardiac markers within 15 minutes using 150 µL of sample.¹⁵ Amic has developed a lateral flow immunoassay device that fills using capillary forces between micropillars.^{16,17} Multiplexed immunoassays have been developed on paper patterned into millimetre wide channels.¹⁸ It would be beneficial to further expand these technologies using even less volume of sample, increasing the sensitivity and accuracy, and measuring more analytes in parallel.

We suggest that a tight control over the volumes and flow rates of sample moved through microfluidic devices gives the opportunity to realize these goals. Our previous work has put emphasis on the development of microfluidics for immunoassays in which capillary forces are used to move liquids in microstructures and antibodies patterned on PDMS provided the specific receptors for the analytes.^{19,20} In this paper, we show the integration of concepts dealing with capillary valves, capillary pumps, and the accurate patterning of cAbs to form capillary driven microfluidics for effecting one-step immunoassays.

The strategy for implementing the concept of the one-step immunoassay chip is illustrated in Fig. 1a. The interaction of analyte, dAbs and cAbs throughout the microfluidic chip is shown in Fig. 1b. Blood or human serum sample is introduced on the blood filter. Cellular components of the sample, such as red blood cells, white blood cells and platelets, and debris are retained by the blood filter membrane and plasma is drawn in the sample collector and into a microfluidic channel. The sample reaches the dAb deposition zone where the dAbs reconstitutes and binds to the analyte. The dAb deposition zone can be a circular microwell or a recessed microchannel for accommodating dAbs spotted there using an inkjet. The analyte-dAb

complex is transported by the moving liquid through the reaction chamber and binds to lines of cAbs on the PDMS cover. Excess dAbs binds to positive control lines of patterned analyte on the PDMS. The sample containing excess analyte and dAbs is drawn by the capillary pump, which largely determines the flow rate and total sample volume used for the immunoassay. A few minutes after the introduction of the sample on the chip most of the dAbs has either bound to cAbs *via* the analyte, bound to the analyte forming the lines for positive control, or was transported to the capillary pumps. In principle, the background fluorescence from dAbs should be low enough to measure the specific detection of analyte on the capture areas, at the intersection of the lines of cAbs and reaction chamber. They can be measured through the PDMS using an external fluorescence reader.

Experimental

Chemicals and biochemicals

PBS tablets (Sigma-Aldrich, St. Louis, MO) were reconstituted in water that was purified using the Simplicity 185 system (Millipore, Billerica, MA) and filtered with a 0.20 µm syringe filter (Sartorius, Epsom, U.K.). Bovine serum albumin (Sigma-Aldrich) was dissolved at a concentration of 1% w/v in PBS. Ethanol (puriss ≥ 99.8%, Fluka, Sigma-Aldrich) was used for cleaning Si chips and PDMS. PDMS Sylgard 184 prepolymers (Dow Corning, Midland, MI) were mixed at a ratio of 1:10 curing agent to polymer using a DOPAG mixer (Cham, Switzerland), poured onto planar polystyrene Petri dishes (Greiner BioOne), and cured overnight in an oven at 60 °C. Cured PDMS was 3 mm thick and cut to the desired size using a scalpel. cAbs were patterned on the surface of the PDMS that had been in contact with the Petri dish.²¹ The blood filters were GF Vivid® plasma separation membranes (PALL, East Hills, NY). Human CRP (8C72), anti-CRP (4C28-C2, 4C28-C6), and human CRP-free serum (8CFS) were purchased from HyTest (Turku, Finland). Human CRP-free serum was spiked with human CRP to the desired CRP concentration. The anti-CRP-C6 Abs were labeled using an Alexa Fluor 647 labeling kit

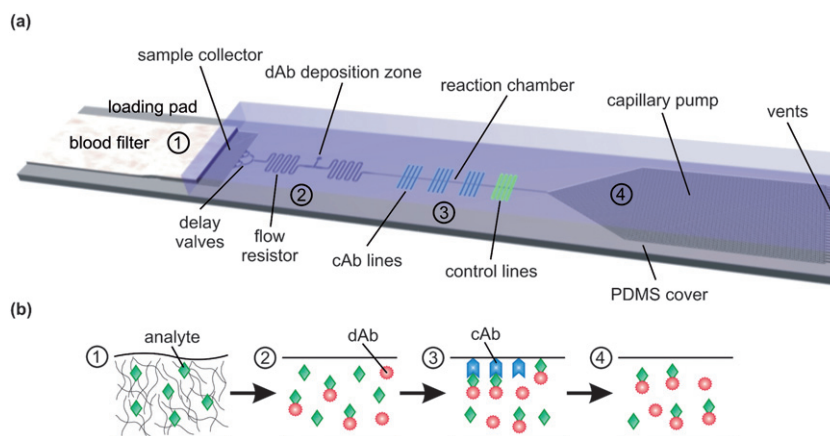


Fig. 1 Concept of a capillary-driven microfluidic chip for effecting immunoassays with one step. (a) A series of functional microfluidic elements are implemented onto the chip for performing immunoassays. (b) The position of and interaction between the analyte, dAbs and cAbs are illustrated along different parts of the chip (see text for details). The PDMS is planar and patterned with lines of cAbs and antigens for the control lines. The Si chip has the loading pad, the sample collector, the delay valves, the dAb deposition zones with dAbs, the reaction chamber, the capillary pumps and the vents.

(Invitrogen, Carlsbad, CA). The fluorophore per protein ratio was measured to be from 3.6 to 3.9 using an Eppendorf BioPhotometer (Hamburg, Germany). Si chips were treated with 1% w/v Pluronic® F108 (BASF, Ludwigshafen, Germany) in purified water. PDMS substrates patterned with cAbs were stored with Sorb-IT silica gel desiccant packets (Sud Chemie, Munich, Germany). Inkjet glass pipettes were cleaned with an alkaline solution of 2% v/v Hellmanex II (Hellma, Müllheim, Germany) in purified water. The solution of dAbs was composed of 250 $\mu\text{g mL}^{-1}$ Alexa 647 labelled anti-CRP-C6, 68 mg mL^{-1} D-(+)-Trehalose (Fluka) and 700 $\mu\text{g mL}^{-1}$ L-phenylalanine (Fluka) in PBS.

Chip fabrication and preparation

The microfluidic chips and stencil templates were micro-fabricated on 4-inch Si wafers (Siltronix, Geneva, Switzerland) using photolithography and photoplotting polymer masks (Selba, Versoix, Switzerland). The microfluidic chips were fabricated by first growing a 0.5 μm thick layer of SiO_2 onto a Si wafer using a wet oxidation process in a thermal furnace (TS-6304, Tempres, Vaasen, The Netherlands). Hexamethyldisilazane (HMDS) was spin coated onto the wafer. Photoresist (AZ 6612, MicroChemicals, Ulm, Germany) was spin coated at 4000 rpm to a thickness of approximately 1.6 μm and soft baked for 1 minute at 110 °C. The first layer mask containing all the features of the microfluidic chip was brought into soft contact with the resist-coated wafer and exposed to a 6.5 mW/cm^2 ultraviolet light for 15 s using a mask aligner (MA/BA6, Süss Microtech, Garching, Germany) and developed. SiO_2 was etched from the features uncovered by the photoresist in buffered hydrofluoric acid (BHF, 7:1 volume ratio of 87.5% NH_4F in water to 12.5% HF in water; danger). The resist was stripped and HMDS was spin coated onto the wafer. A layer of AZ 4562 (MicroChemicals, Ulm, Germany) was spin coated at 4000 rpm to a thickness of approximately 7 μm and soft baked for 1 minute at 110 °C. The second layer mask containing all the features of the microfluidic chip except the reaction chamber was photoexposed for approximately 45 s and developed. Deep reactive ion etching (AMS-200SE, Alcatel Micro Machining Systems, Annecy Cedex, France) was used to etch the second layer features in Si to a depth of 160 μm . The resist was stripped and the first layer features, containing all the features of the microfluidic chips, were etched to a depth of 20 μm using reactive ion etching. The total depth of features was 20 μm in the reaction chamber and 180 μm elsewhere. The remaining SiO_2 was removed in buffered hydrofluoric acid in approximately 5 minutes.

Deep reactive ion etching was used to transfer features and etch through Si wafers to make the stencils. Si wafers with stencils were diced into 12 \times 12 mm^2 individual templates having 4 groups of 4 stencils. Each stencil comprised of a loading pad connected to a 2-mm-long and 100- μm -wide line etched through the wafer. The microfluidic chips were cleaned using an O_2 -based plasma (Tepla microwave-plasma system 100, PVA Tepla, Asslar, Germany) for 2 min at 200 W and 0.7 Torr. The microfluidic chips were placed in a 1% solution of Pluronic® F108 for 30 min, rinsed with purified water and dried under an N_2 stream. The chips were stored in a dark and dry environment and used within 4 h.

Capture antibody patterning on PDMS

Stencil templates were cleaned using an O_2 -based plasma. Stencils were fabricated using single side polished Si wafers. The polished side of the stencils was placed onto PDMS substrates. Stencils were filled with a solution of 125 $\mu\text{g mL}^{-1}$ anti-CRP-C2 in PBS for a 15 min long deposition of cAbs on PDMS at room temperature. To prevent evaporation of the cAbs in the stencils, the deposition was undertaken in a humidity chamber consisting of a small Petri dish with sheets of clean room paper soaked in water on the bottom. The stencils were rinsed under a stream of PBS and purified water. PDMS substrates were separated from the Si stencil template using tweezers in the presence of a blocking solution of bovine serum albumin where they were incubated for 15 min. The regions of the PDMS not patterned with cAb were passivated with bovine serum albumin. The PDMS substrates were then rinsed with a stream of PBS, purified water and dried under a stream of N_2 . Templates were rinsed in purified water, ethanol, and cleaned with O_2 plasma before reuse.²¹ The PDMS substrates were placed onto microscope slides with the patterned side facing upwards. The substrates were stored in N_2 purged 50 mL Falcon conical tubes (BD Biosciences, San Jose, CA) in the dark. A Silica gel packet was inserted into some of the conical tubes. These substrates were used for the study of the lifetime of cAbs on PDMS or for exploring the performances of one-step immunoassays, in which case they were used within 3 days.

Study of the lifetime of cAbs on PDMS

A PDMS substrate having lines of cAbs was placed on a microfluidic chip with the lines of cAbs perpendicular to the reaction chambers. The chip covered with the PDMS substrate was then placed into a humidity chamber to prevent evaporation of the samples in the loading pads. These immunoassays were performed on multistep chips that had 6 independent flow paths.²¹ One μL of human serum spiked with CRP was pipetted into the loading pads, followed by 0.5 μL of 250 $\mu\text{g mL}^{-1}$ dAbs and 1 μL of CRP-free human serum for rinsing. After 14 min, the PDMS was removed from the microfluidic chip, rinsed under a stream of PBS, rinsed with purified water, dried under a stream of N_2 , and the fluorescent signals on the PDMS were imaged in the fluorescence microscope.

One-step immunoassay

The solution of dAbs was deposited in the microfluidic chips using an Autodrop MD-P-705-L (Microdrop, Norderstedt, Germany) inkjet printer and a AD-K-501 piezo-driven pipette having a nozzle of 70- μm in diameter. The pipette was first cleaned with Hellmanex® II (Hellma, Müllheim, Germany) alkaline solution for two hours and rinsed with purified water in order to remove possible residues from previous experiments. The inkjet pipette nozzle was positioned over the detection antibody deposition zone where 20 drops of 180 pL each were deposited for a total of 3.6 nL of detection antibody solution. Solutions of dAbs can be inkjet deposited on the surface of a chip next to the circular well. In this way, there are dAbs in the channel and on the surface of the chip between the Si and PDMS substrate. This further slows down the redissolution of dAbs in

the sample and increases the detection signal. This deposition method was used for immunoassays on the one-step chips. After deposition the microfluidic chip was stored in the dark and used within 4 hours. The conditions for jetting the drops and their geometry were optimized using a camera and stroboscopic viewing system embedded in the inkjet instrument. Ten μL of human serum was used to characterize the filling behaviour of the chip. A blood filter was placed in such a way that the interface with the chip covered about half of the serum collector. A paper clip was used to provide sealing pressure between the filter and the serum collector with the other half and the rest of the microfluidic chip covered with a piece of bovine serum albumin passivated PDMS. For the one-step experiments one or several PDMS pieces, one of which having lines of cAbs, were placed on the microfluidic chips. The microfluidic chip covered with PDMS was then placed on the stage of a fluorescence microscope. The immunoassays started when 5 μL of human serum were pipetted into the loading pad of a microfluidic chip. Fluorescence in the reaction chamber was monitored directly by viewing through the PDMS. After the experiments, the microfluidic chips were rinsed with purified water, ethanol, dried under a stream of N_2 , and cleaned using an O_2 -based plasma and reused in further experiments.

Instrumental setup and data acquisition

High-resolution fluorescence micrographs (using $\times 10$ objectives) were obtained using a fluorescence microscope (Eclipse 90i, Nikon, Japan). Excitation of fluorophores was done using a 100 W halogen lamp and images were taken using a black and white CCD camera (DS-1QM, Nikon) cooled to -30°C . The software NIS-Elements (Nikon) was used for analysis of the fluorescence images. Videos were composed by joining a series of time-lapse images.

Results and discussion

An example of a one-step microfluidic chip is shown in Fig. 2. This chip is in Si but other materials can be used. This layout can be replicated in materials such as cyclic olefin copolymer, poly(methylmethacrylate) and polycarbonate using mold injection or hot embossing with a low cost of fabrication and to make the

chips disposable after each test. The microfluidic chip was treated with an aqueous solution of Pluronic[®] F108. Pluronic[®] F108 is a block copolymer of the formula $\text{HO}-(\text{CH}_2\text{CH}_2\text{O})_{133}-\text{C}(\text{CH}_3)(\text{CH}_2\text{O})_{50}-\text{C}(\text{CH}_3)(\text{CH}_2\text{O})_{133}-\text{H}$ which is used as a surfactant and a wetting agent. It is not the goal of this paper to investigate in detail how Pluronic[®] F108 interacts with hydrophobic and hydrophilic surfaces²² but we noticed that after microfluidic chips were cleaned with oxygen plasma and treated with aqueous Pluronic[®] F108, the contact angle of human serum with the chip increased from 0° to 20° (with a receding contact angle of 0°). This characteristic was highly reproducible and the surface was stable for at least 4 hours when the chip was exposed to ambient conditions. We therefore decided to use this method of treatment over coating the Si chip with gold that was subsequently derivatized with thiolated polyethylene glycol.²¹

The first microfluidic structure in the chip is the sample collector shown in Fig. 2a. It is 180 μm deep and has elongated posts arrayed in a centered rectangular lattice. The vertical and horizontal distances between posts from edge to edge are 30 μm and the capacity of the sample collector is 2 μL . The capillary pressure exerted on a liquid in the sample collector ranges from -1.33 kPa to -2.94 kPa between posts and is very good at wicking the liquid out of the Vivid[®] plasma separation membrane, composed of microstructured asymmetric polysulfone, down into the sample collector. The sample does not always reach the end of the sample collector with a straight filling front. For this reason, streams of sample are collected using delay valves.²³ These valves start with two inlets merging into one outlet and consolidate incoming streams of sample into one. A meniscus progressing through one inlet of a delay valve stops until it can merge with the meniscus of an adjacent inlet. The menisci of the filling fronts move from regions of high capillary pressure (30 μm wide channels) into regions of reduced capillary pressure (45 μm and 60 μm wide channels). The channel width is gradually increased at each level to keep friction low and the flow rate similar. For example, from 5 levels of delay valves we can collect liquid from a 1900 μm filling front into a 60 μm wide channel without generating air bubbles. Once in the sample collector, the sample (here serum) needs only a few seconds to reach the delay valves and merge into a 60 μm wide channel.

The next set of microfluidic structures are flow resistors and a zone for depositing dAbs shown in Fig. 2b. The key concept to

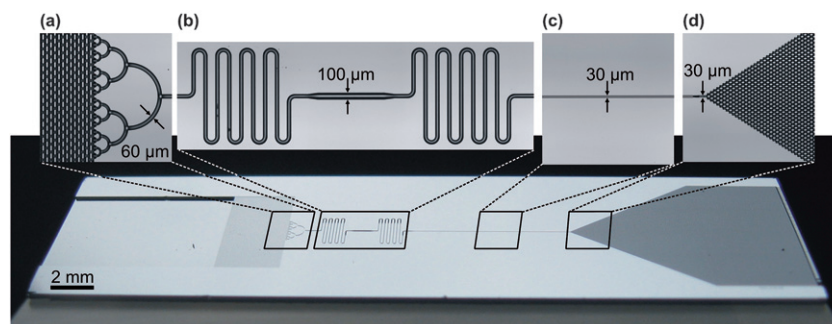


Fig. 2 Optical photograph of the microfabricated Si microfluidic chip for one-step immunoassays having as main functional elements: (a) sample collector ending with hierarchical delay valves, (b) flow resistors and central deposition zone for dAbs, (c) reaction chamber and (d) capillary pump. All microfluidic elements are 180 μm deep except for the reaction chamber that is 20 μm deep. The chip is 43×11 mm^2 and has an average flow rate of filling with human serum of 82 nL min^{-1} .

implementing a one-step immunoassay is to have all reagents on-chip. Solutions of dAbs are deposited onto the chip in a zone that is 180 μm deep and 100 μm wide using an inkjet that generates drops with a diameter of 70 μm . The deposition zone defines the redissolution profile of the dAbs when the assay is performed. Flow resistors are meandering microchannels that can be used to store overflow quantities of dAbs deposited in the deposition zone and prevent them from reaching the serum collector or the reaction chamber. The width, length and repeat number of curves can be varied to change the hydraulic resistance of the flow resistors. This can be used to change the redissolution profile of dAbs and the reaction kinetics of immunoassays that target different reagents or sensitivity levels. The flow resistors are 180 μm deep and 60 μm wide and have a capillary pressure of -1.92 kPa. The flow resistors and deposition zone have a total volume of 300 nL. The flow resistor is a convenient feature which can be changed at will to modify the hydraulic resistance of the entire microfluidic chip. This prevents having to redesign other more complex parts of the chip if chips requiring different flow rate characteristics must be fabricated.

The reaction chamber is shown in Fig. 2c. A PDMS substrate, patterned with lines of cAbs and lines of analyte, is placed onto the reaction chamber so that the lines are perpendicular to the main axis of the reaction chamber. The detection signal and positive control can be measured through the PDMS substrate in the reaction chamber using a fluorescent microscope. The redissolution of dAbs can also be observed in this way. The reaction chamber is 30 μm wide and 20 μm deep with a capillary pressure of -6.22 kPa and a total volume of 50 nL. The reaction chamber is shallower than the other parts of the chip for accelerating the immunoassay reaction by providing short diffusion distances for the analyte-dAb complex to bind to the cAbs on the PDMS substrate.

The capillary pump shown in Fig. 2d contains rounded hexagonal posts arrayed in a centered rectangular lattice with a depth of 180 μm . The rounded hexagonal posts are used to define a reproducible filling behaviour that minimizes the formation of air bubbles.²⁴ The distance between hexagonal posts is between 15 μm and 40 μm and the capillary pressure accordingly varies from -2.80 kPa to -7.20 kPa. The total volume of the pump is 10 μL . The capillary pump adds only a small flow resistance because it is composed of many parallel flow paths. Within a minute after the addition of the sample onto the loading pad, the sample fills the chip up to the capillary pump. The capillary pump has the largest capillary pressure on the chip and it determines the flow rate together with the flow resistor, and the maximum volume of sample used in the immunoassay. The area of the pump that was filled in a given time interval determined the average flow rate, which was in the case of human serum 82 nL min^{-1} during a 14 minute one-step assay.

PDMS does not seem to be commonly used for disposable labware in biology although it is frequently employed in research. This is somewhat surprising because first, PDMS exhibits the lowest autofluorescence across the visible spectrum of many plastic and glass materials commonly used for biological assays.²⁵ Second, PDMS is chemically stable and highly transparent. Third, it is hydrophobic and therefore is easy to coat with proteins by simple adsorption from solution. Fourth, immunoassays performed on PDMS substrates can achieve picomolar

sensitivity.²⁶ An essential question is whether it is suited for use in biological assays after being patterned with proteins and stored for large periods of time. The lifetime of cAbs on PDMS is probed by performing fluorescence immunoassays using human serum spiked to different concentrations of CRP: 1 $\mu\text{g mL}^{-1}$, CRP free serum, 100 ng mL^{-1} , 30 ng mL^{-1} , 10 ng mL^{-1} , and 1 ng mL^{-1} , one concentration per horizontal reaction chamber from top to bottom, Fig. 3.²¹ The PDMS substrates were patterned with lines of cAbs perpendicular to the reaction chambers and stored at room temperature without and with desiccant for up to 6 months. The micrographs in Fig. 3a and b show the measured fluorescent signals corresponding to the different concentrations of CRP in the serum for either storage condition. An important loss of signal is observed after only two weeks when no desiccant was used, Fig. 3a. After 6 months, the signal measured for a concentration of CRP of 1 $\mu\text{g mL}^{-1}$ falls to approximately 14% of the signal measured on PDMS freshly patterned with cAbs. This contrasts markedly with results obtained when the patterned PDMS substrates were stored with desiccant. In the latter case, the signal measured on 6 month old substrates corresponds to 51% of the signal measured on fresh substrates for a CRP concentration of 1 $\mu\text{g mL}^{-1}$. The fluorescence

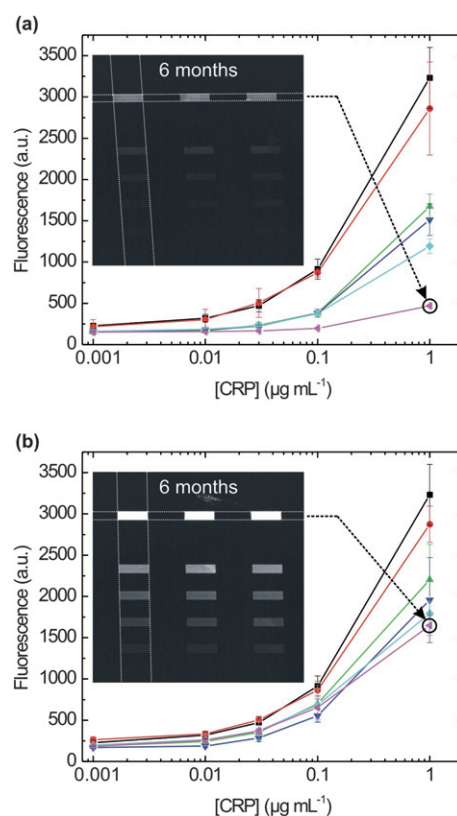


Fig. 3 Study of the lifetime of cAbs on PDMS when stored at (a) room temperature and (b) room temperature with desiccant. Immunoassays were performed with human serum spiked with CRP and a capillary microfluidic chip that had 6 parallel reaction chambers (horizontal direction). The PDMS samples that had patterned cAbs (vertical direction) were (■) fresh or stored for (●) 1 week, (▲) 2 weeks, (▼) 1 month, (◆) 3 months, and (◄) 6 months. The fluorescent micrograph shows the strength of the fluorescent signal of PDMS substrates stored for six months (see text for details).

microscope image in Fig. 3b even reveals that signals down to a concentration of 10 ng mL^{-1} are easily detected.

These results are important as they clearly indicate that PDMS can be used a long time after it has been patterned with cAbs and that similarly to proteins immobilized on other plastics (*e.g.* polystyrene) cAbs have a long lifetime when stored under dry conditions. Here, the high mobility of siloxane chains at room temperature and the presence of non crosslinked and/or low molecular weight species in PDMS do not seem to interfere with the biofunctionality of the layer of cAbs. Dry conditions favour the dehydration of proteins and limit molecular mobility and rearrangements of their constituents thereby making (oxidative) degradation of proteins less likely. We suggest in addition that the lifetime could even be further augmented by adding stabilizing agents in the solution pipetted in the stencils. These agents typically have multiple hydroxyl groups that stabilize hydrophilic amino acids on protein shells.²⁷

The key to one-step immunoassays is having all reagents for the immunoassay on-chip. In previous work, we pipetted by hand “large” volumes of dAbs of 150 to 500 nL on chips and shock froze them.²⁸ Here the deposition of dAbs is greatly simplified. Volumes of dAbs 40 times smaller are used and the deposition zone of the chip can be kept small compared to the rest of the chip owing to the precise spotting of solutions of dAbs using an inkjet. Deposition zones for dAbs determine the volume of dAbs that can be deposited and their redissolution profile when the immunoassay is performed. Three deposition zones for dAbs were tested, Fig. 4a. The first deposition zone was the simplest and consisted of a slightly enlarged channel between a set of flow resistors. Solutions of 3.6 nL of dAbs deposited in

this deposition zone using an inkjet redissolve as a plug that completely passes through the reaction chamber within one minute. A sensitivity for CRP of 100 ng mL^{-1} was obtained within 2 minutes after sample introduction onto the loading pad. This deposition zone can be used for immunoassays of higher concentration samples that require quick time to results within a few minutes. In order to increase the sensitivity, the sample should preferably redissolve the dAbs into a larger plug to allow more time for forming the analyte-dAb complex and capturing them. The second deposition zone consists of a side channel ending with a circular well where 3.6 nL (20 drops) of a solution of dAbs was deposited. The circular well had a diameter of $200 \mu\text{m}$ to provide a large enough area for depositing $70\text{-}\mu\text{m}$ -diameter drops of dAbs using an inkjet microdispenser. When the sample fills the chip, it enters the side channel and progressively fills it thereby redissolving the dAbs. The liquid slows down at the edge of the circular well due to the entrapment of air there. This results in a depletion of the deposited dAbs that dissolve in the liquid, diffuse passively from the side channel to the main channel and are transported by the sample flowing toward the reaction chamber. Analyzing the intensity profile of the fluorescence in between the capture areas over time, we found that 90% of the dAbs dissolved in a sample volume of 850 nL. This geometry led to a sensitivity below 1 ng mL^{-1} and was employed throughout the rest of the paper. The third deposition zone adds horizontal channels for depositing larger volumes of dAbs and for enabling longer incubation times. Sample slowly fills one horizontal channel at a time, entrapping air at each circular well. Then a large air bubble is formed in the entire deposition zone that propagates and blocks the main channel, creating a stop

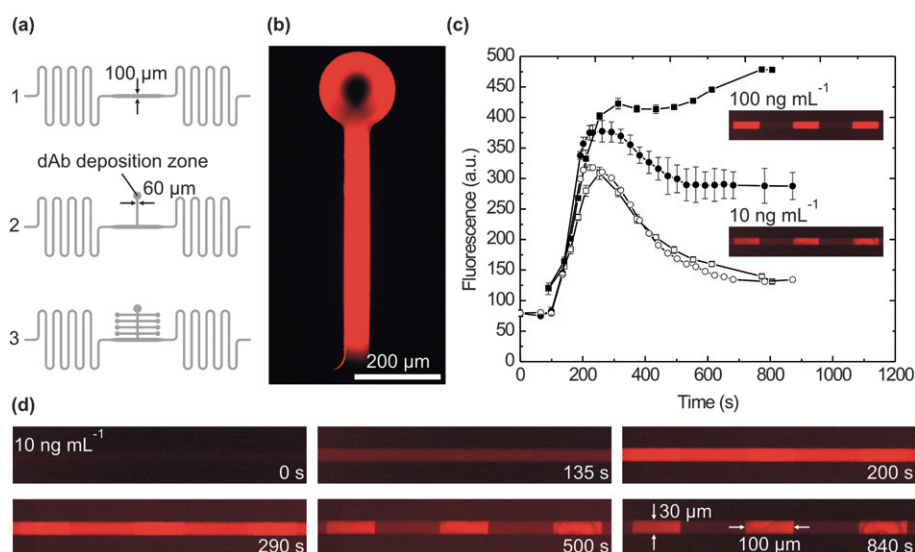


Fig. 4 Deposition of dAbs on the microfluidic chip and its influence on the immunoassay. (a) The 3 layouts show examples of zones for depositing solutions of dAbs. This zone can be designed so as to vary the redissolution profile of dAbs and the vicinal flow resistors can be adjusted to define the flow rate in the microfluidic chip. (b) This fluorescence micrograph reveals the distribution of Alexa 647-labelled solution of dAbs, which was deposited in a zone as in a2 using an inkjet (20 drops \times 3.6 nL) and dried. Such a deposition zone was used throughout the paper. (c) The fluorescent signal measured on (specific signal) and in between (background signal) capture areas varies over time with the capture of analyte-dAb complex and the flow of dAbs in solution. The fluorescent intensity of capture areas is shown for (■) 100 ng mL^{-1} and (●) 10 ng mL^{-1} and the corresponding background signal outside of capture areas for (□) 100 ng mL^{-1} and (○) 10 ng mL^{-1} . The shown fluorescence micrographs were taken approximately 14 min after adding the sample on the chip. (d) Fluorescence time lapse micrographs of 3 capture areas showing the increasingly visible detection of CRP from human serum that was spiked to 10 ng mL^{-1} .

valve. When enough air permeates through the PDMS, after more than 40 minutes, the main channel is unblocked and the sample plug reaches the reaction chamber. It was not possible to measure CRP at a concentration of 1 ng mL^{-1} within 30 minutes using the third deposition zone. Reducing the number of horizontal channels in a future design might be used to entrap less air and reduce the time required for air to permeate through the PDMS. In this way, higher sensitivities could be achieved with a programmed incubation time of the analytes with dAbs.²⁹

Inkjet printers are very precise and can be aimed within small regions of the chip as shown in Fig. 4b. The total concentration of protein had to be approximately a maximum of 1 mg mL^{-1} in order to minimize viscosity and allow for the reproducible formation of droplets using the inkjet. The concentration of dAbs was $250 \text{ } \mu\text{g mL}^{-1}$ and L-phenylalanine was $700 \text{ } \mu\text{g mL}^{-1}$. L-phenylalanine was used to increase the total concentration of proteins in the dAb solution because proteins are more stable at higher concentrations. Additionally, L-phenylalanine creates a scaffold when dried that helps immobilize the dAbs and prevent denaturation. Trehalose and other sugars such as sucrose and glucose, are often used in protein solutions that are inkjet deposited and dried. These sugars help stabilize the proteins and slow down denaturation.²⁷ Leaving the chip to dry in air after deposition using the inkjet was sufficient to provide a good particle free redissolution provided the trehalose concentration was high enough. There was no need for freeze-drying. Solutions of dAbs with a trehalose concentration of 25 mM redissolved with many particles and protein aggregates. The concentration was increased to 50 mM , 100 mM and 200 mM with the latter leading to the complete suppression of particles in the redissolution and thus it was used throughout the experiments. The microfluidic chip fabrication, inkjet deposition of dAbs, cAb patterning on PDMS and assembly into a device would be manufactured, shipped to the hospital and stored before use.

The evolution of the fluorescent signals measured through the PDMS over the reaction chamber for two separate chips after the addition of a sample with a concentration of CRP of 10 ng mL^{-1} and 100 ng mL^{-1} is shown in Fig. 4c. The curves obtained from the background signals measured between the capture areas are very similar for both chips. The deposition and redissolution of dAbs between two separate chips is reproducible. The specific signals measured on the rectangular capture areas contain the signal from analyte-dAb complexes on the cAbs and the background signal of nearby dAbs in solution. Micrographs taken from the timelapse video of the detection of a concentration of CRP of 10 ng mL^{-1} are shown in Fig. 4d. The dAbs are gradually redissolved and pass through the reaction chamber to reach a peak in background signal at 250 s after which the number of dAbs in the sample gradually decreases and the specific signal becomes increasingly visible. The capture areas are homogenous and small with dimensions of $30 \text{ } \mu\text{m}$ by $100 \text{ } \mu\text{m}$. Three capture areas occupy only a length of $600 \text{ } \mu\text{m}$. These capture areas can be patterned with up to 16 different cAbs to provide multiplexed detection of several analytes and with different patterning there is no need to use different fluorescent markers. In addition, the dAb deposition zone is large enough to accommodate larger amounts of dAbs without redesigning the chip. A supplementary video shows the evolution of the fluorescent signal from dAbs in the reaction chamber and the increase of the signal on the PDMS

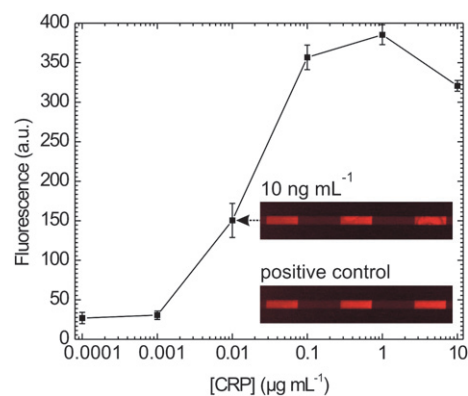


Fig. 5 Graph of the fluorescent signal obtained for immunoassays done with different CRP concentrations on the one-step chip. The fluorescence micrographs respectively correspond to the signal and positive control of detection of CRP from human serum that was spiked to 10 ng mL^{-1} obtained 14 min after adding the sample on the chip.

substrate after the addition on the chip of human serum spiked with CRP at a concentration of 1 ng mL^{-1} .†

The graph shown in Fig. 5 was plotted from the detection signal obtained on separate one-step chips 14 minutes after the addition of sample. The sensitivity was at least 1 ng mL^{-1} . The micrographs show a clear specific signal for a concentration of CRP of 10 ng mL^{-1} and a positive control signal on the same chip where dAbs bind to CRP on the PDMS surface. Above $1 \text{ } \mu\text{g mL}^{-1}$ CRP the high-dose hook effect is observed.³⁰ The excess analyte saturates both the dAbs and cAbs and prevents to some extent the analyte-dAb complex from binding to the cAbs on the PDMS substrate. This commonly happens in solid phase assays when there are limited quantities of dAbs. Adding more dAbs would lead to a flattening of the curve at high concentrations. Implementing positive control lines after the capture areas provides two benefits. First, a user can verify that the sample has filled the reaction chamber and passed the capture areas up to the capillary pump indicating that the chip has filled properly. Second, the stability, activity and redissolution of dAbs can be measured and test results can be interpreted as non-valid or valid based on the normality of the positive control signal. A doctor or nurse may use the device by adding $5 \text{ } \mu\text{L}$ of patient sample (blood or serum), waiting 15 minutes and inserting the device into a fluorescence reader. The device would be ideal for clinicians who tend to observe the evolution of CRP levels in the blood of patients to perform prognostics: for example, an increase in concentration of CRP in a patient's serum can indicate a worsening of sepsis³¹ or a diminution can indicate a recovery after an acute myocardial infarction.^{32,33}

Conclusions

The one-step chip presented here provides many features. The chip is autonomous and requires only the addition of a drop of sample. The sample is merged seamlessly from several parallel filling fronts into a single channel by delay valves without forming air bubbles. Nanoliter volumes of dAbs are incorporated on-chip and redissolved without particles. Sixteen lines of cAbs and analytes for positive control are patterned onto

a PDMS cover. CRP is detected at a concentration below 1 ng mL⁻¹ in 5 minutes using 5 µL of human serum. The use of a peripheral fluorescence reader keeps active components outside of the chip and provides sensitive concentration measurements of analytes for patients' digital health record that should help doctors use test results in standard operating procedures and administer the appropriate treatment. The main task that remains is to fabricate the chip using plastic components to create a disposable chip for use in hospitals and elsewhere. This should however not be difficult considering the capabilities of plastic engineering using injection molding or hot embossing.

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