PAPER

High-sensitivity miniaturized immunoassays for tumor necrosis factor α using microfluidic systems†

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We use microfluidic chips to detect the biologically important cytokine tumor necrosis factor α (TNF-α) with picomolar sensitivity using sub-microliter volumes of samples and reagents. The chips comprise a number of independent capillary systems (CSs), each of which is composed of a filling port, an appended microchannel, and a capillary pump. Each CS fills spontaneously by capillary forces and includes a self-regulating mechanism that prevents adventitious drainage of the microchannels. Thus, interactive control of the flow in each CS is easily achieved *via* collective control of the evaporation in all CSs by means of two Peltier elements that can independently heat and cool. Long incubation times are crucial for high sensitivity assays and can be conveniently obtained by adjusting the evaporation rate to have low flow rates of $\sim$30 nL min^{-1}. The assay is a sandwich fluorescence immunoassay and takes place on the surface of a poly(dimethylsiloxane) (PDMS) slab placed across the microchannels. We precoat PDMS with capture antibodies (Abs), localize the capture of analyte molecules using a chip, then bind the captured analyte molecules with fluorescently-tagged detection Abs using a second chip. The assay results in a mosaic of fluorescence signals on the PDMS surface which are measured using a fluorescence scanner. We show that PDMS is a compatible material for high sensitivity fluorescence assays, provided that detection antibodies with long excitation wavelength fluorophores ($\geqslant$580 nm) are employed. The chip design, long incubation times, proper choice of fluorophores, and optimization of the detection Ab concentration all combine to achieve high-sensitivity assays. This is exemplified by an experiment with 170 assay sites, occupying an area of $\sim$0.6 mm^2 on PDMS to detect TNF-α in 600 nL of a dendritic cell (DC) culture medium with a sensitivity of $\sim$20 pg mL^{-1} (1.14 pM).

1. Introduction

The miniaturization of biological assays currently enabled by novel chemistries and microtechnologies is an important trend motivated by the need to use expensive reagents and precious samples sparingly, and to analyze multiple liquids in parallel.[1–3] We pursue the task of miniaturizing assays by devising microfluidic chips to effect surface immunoassays. Immunoassays rely on the specific interactions between antigens and Abs to detect analyte molecules (antigens) with high sensitivity. We take immunoassays in which Abs are first immobilized on a surface, and use them to capture analyte molecules in solution, after which fluorescently-tagged Abs binding to the captured analyte molecules quantify the amount of analyte molecule on the surface. This type of immunoassay corresponds to a two-site (or sandwich) fluorescence immunoassay. Common surfaces for these assays are the polystyrene wells of a microtiter plate, micrometer-sized beads suspended in solution, or microscope slides derivatized, for example, with an array of various Abs. Our method employs PDMS as the assay surface and a microfluidic chip to localize the delivery of one or more of the solutions necessary for the assay onto a small area of the PDMS.[4,5]

The features of microfluidics that can impact (immuno)assays the most are (i) the economical use of samples and reagents by using sub-microliter volumes of solution, (ii) the fast transport of reactants onto the substrate for the assay, (iii) the control over evaporation of small volumes of solution that may otherwise dry out quickly and may thus denature biomolecules or change the concentration of dissolved analyte molecules, and (iv) the integration of microstructures for mixing, transporting, and analyzing liquids.[6–8] In general, it is desirable to develop microfluidic-based immunoassays using small form-factor platforms that enable highly sensitive and specific assays.[4,9–12] These immunoassays could be applied to research in the life sciences and *in vitro* diagnostics.[12,13] Our approach to miniaturizing immunoassays is to use capillary systems (CSs) to localize all the assay steps on a few square micrometers of a PDMS surface while using microliter volumes of solutions or less.[14]

We define a CS as one functional microfluidic unit that uses capillary forces to displace and process liquids.[15] Independent CSs can be arranged to form an array in a Si chip, where each CS can accommodate microliter aliquots of solution and have one or more valves and an integrated capillary pump. In this paper, we extend the concept of CSs to immunoassays having picomolar sensitivity and show that the resulting assays are combinatorial, fast, and amenable to detecting analyte molecules with minimal sample volumes. We illustrate the practicability of this approach to detect the cytokine TNF-α from buffers and various cell culture supernatants.

Cytokines are regulatory proteins that act as a kind of hormone and are mainly produced by cells of the immune system. These proteins are important physiological and pathological markers for the prognosis and diagnosis of diseases as well as for research applications. TNF-α is a

† Electronic supplementary information (ESI) available: surface treatment of chips; background fluorescence of PDMS substrates. See http://www.rsc.org/suppdata/lc/b4/b408964b/

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pleiotropic factor capable of activating multiple signal transduction pathways and modulating the expression of a wide variety of genes.[16] It plays many different roles in the generation and regulation of inflammation and immune responses. TNF-α is critically involved in the pathogenesis of several chronic inflammatory diseases such as rheumatic arthritis and Crohn's disease.[17] Cytokines are most often measured using sandwich ELISAs, either in cell culture supernatants, serum or plasma. Cytokine levels, like TNF-α, tend to be low in healthy patients, usually at pg mL^{-1} concentrations, and thus require high-sensitivity assays. In pathological conditions TNF-α levels are elevated ten to one hundredfold, which makes the detection of TNF-α relatively easier.[18] In the experiments described in this paper we detect cytokines secreted by immature and mature DCs.

Fig. 1 shows a chip having an array of 11 independent CSs mounted on a microfluidic workstation (Fig. 1a) and the steps necessary to perform a surface immunoassay (Fig. 1b). The chip–user interface of each CS is a millimeter-sized filling port. A drop of solution that is pipetted into a filling port spontaneously fills the appended microchannel and is drawn through the system by a capillary pump. The volume of solution that can be loaded in a filling port in the CS shown is ~300 nL, but deeper and larger ports can also be made to accommodate larger volumes. The lowest usable volumes that can be pipetted manually are in the range of ~100 nL. The flow speed of the liquid inside the microchannel can be tuned by modifying the design of the CS, which affects the capillary pressure and flow resistance, and by using "active evaporation" during the assay when the pump is full. Active evaporation is achieved by controlling the temperature of the capillary pumps relative to the fill ports using two Peltier elements. The Peltier element beneath the fill ports is cooled just above the dew point, in order to minimize evaporation and thus to prevent concentrating samples. The capillary pumps are held at a certain temperature above the dew point. Flow rates below 10 nL min^{-1} and up to 500 nL min^{-1} are achieved using this method. For higher flow rates, the capillary pump can also be relayed with a secondary capillary system, *e.g.* a tissue or filter paper that wicks away the contents of the flow promotion structures. The temperature transition zone is narrow, as can be seen in Fig. 1 where the right side of the chip was cooled below the dew point for illustration. In the experiments done here, the temperature was always maintained above the dew point to avoid condensation, which otherwise might cause dilution of the samples.

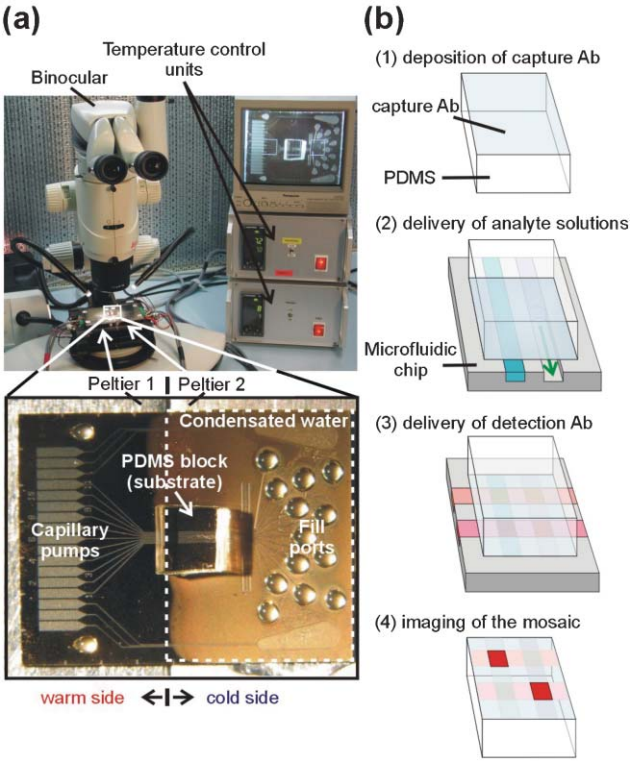

Fig. 1 (a) Microfluidic workstation used to perform highly miniaturized surface immunoassays. The workstation comprises a binocular equipped with a digital camera, a chip carrier having two Peltier elements and control units, and the chip itself. The chip visible in the inset has eleven independent CSs, the filling ports of which are filled with ~0.3 μL of solution. The microchannels of all CSs converge in a region over which a PDMS substrate is placed to localize the assays. (b) Capture Abs and BSA blocking proteins are deposited everywhere on the PDMS, but the analyte molecules and detection Abs are locally flushed over the PDMS surface. The formation of a sandwich comprising the capture Ab, the analyte molecule, and the detection Ab occurs on sites forming a combinatorial matrix (mosaic) of binding events.

The substrate for the assay is a block of PDMS with a surface area of 5 × 5 mm^2 that seals the region where the 30 μm wide microchannels of each CS converge and run parallel. The microchannels before and after the PDMS block are, like the other structures of the chip, open to the atmosphere. PDMS is a hydrophobic elastomer that spontaneously seals the parts of a CS that it covers by conformal contact.[19] PDMS can be coated with capture Abs or other proteins by adsorption from solution.[20–22] In the experiments described here, PDMS is precoated with monoclonal Abs against TNF-α, which act as the capture molecules, Fig. 1b. This is followed by a blocking step with BSA. The PDMS block is then placed on an array of CSs to capture analyte molecules from solutions forming lines and then on a second array of CSs, to deliver the detection Ab solutions. The microfluidic-based capture step is the key to miniaturizing the assay, whereas the second step is optional but allows us to screen for various analyte molecules in parallel or to vary other parameters of the assay.[14,21] The exposure of capture Abs to crossing solutions of analyte and detection Abs results in a micromosaic of fluorescent signals.[21] Another advantage of this format is that $n \times m$ reaction zones can be defined using only $n + m$ pipetting steps.

2. Materials and methods

2.1. Proteins and reagents

Chemicals were obtained from Fluka and proteins from Sigma unless indicated otherwise. Mouse anti-human TNF-α monoclonal Ab (clone MAb 1, capture Ab) and biotinylated as well as non-biotinylated mouse anti-human TNF-α monoclonal Ab (clone MAb 11, detection Ab) were obtained from BD Biosciences PharMingen (San Diego, USA). Deionized water (produced with a Simplicity 185 System, Millipore) was used throughout this work. Solutions of phosphate-buffered saline (PBS) (BuPH, Pierce, Rockford, IL), 1% bovine serum albumin (BSA) in PBS and 0.05% Tween 20 in PBS were filtered through a 0.22 μm filter before pipetting them into filling ports.

Antibodies used for capture were diluted in carbonate buffer (0.5 M Na_2CO_3, pH 9.6). Recombinant human TNF-α (used as standard) was from R&D Systems (Minneapolis) and diluted in 1% BSA in PBS. Anti-human TNF-α detection Ab was labeled with Alexa 647 (Alexa Fluor® 647 mAb Labeling Kit, Molecular Probes) according to the recommendations of the manufacturer. The resulting Abs had 1 mole dye per 1 mole protein on average.

A thiolated poly(ethyleneglycol) (HS-PEG, 12750-4, Rapp Polymere, Tübingen, Germany) and eicosanethiol ((HS–$(CH_2)_{19}$–CH_3, Robinson Brothers, West Bromwich, UK) were diluted in absolute ethanol.

2.2. Capillary systems

The ports and microchannels of the chips were etched into a 4 inch silicon wafer to a depth of 20 μm using reactive ion etching (AMS 200, I-speeder, Alcatel Vacuum Technology, Annecy, France) and a patterned photoresist as the mask. The chips were then coated with 10 nm of Ti (adhesion layer) and 50 nm of Au using a sputtering evaporator (LA440S, VonArdenne Anlagetechnik GmbH, Dresden). Covering the chips with Au allows their surface to be derivatized with a hydrophobic monolayer of eicosanethiol or a hydrophilic, protein-repellent monolayer of HS-PEG (see electronic supplementary information†).[14,23] After each use, the chips were immediately rinsed with PBS, sonicated in water for up to 8 min, rinsed with ethanol, and dried. Before reusing a chip, we removed the monolayers and made new ones. The monolayers were removed by exposing the chips to either an O_2-based plasma (O_2 pressure 0.1–0.5 mbar, load coil power $\sim$200 W, exposure time 30–60 s, Technics Plasma 100-E, Florence, KY, USA) or to ozone (UV-Ozone Photoreactor PR-100, Ultra-Violet Products, Upland, CA) for 30 min.

2.3. PDMS substrates

PDMS substrates were prepared by dispensing the Sylgard 184 prepolymers (Dow Corning, Midland, MI) with an automatic mixer/dispenser (DOPAG Micro-Mix E, Cham, Switzerland) on the planar polystyrene surface of a Petri dish (Falcon 1001 & 1013, Becton Dickinson Labware, NJ) and cured at 60 °C for 24 h. PDMS blocks of approximately 5 × 5 mm^2 were cut from a 3 mm thick layer of PDMS and served as the substrate for immunoassays. The background fluorescence of the PDMS substrates was investigated (see electronic supplementary information†) in order to optimize the choice of the fluorescent marker.

2.4. Miniaturized sandwich immunoassays

PDMS substrates were coated with capture Abs by separating two blocks of PDMS using a 0.17 mm thick glass spacer and filling the interstice with $\sim$2 μL of a 100 μg mL^{-1} solution of anti-TNF-α Ab for 1–2 h at RT. The PDMS blocks were rinsed for 10 s with a solution of 1% BSA in PBS, then only with PBS, and for 3 s with deionized water, and dried with N_2. The PDMS block coated with the capture Ab was placed on the central region of the microchannels. All subsequent operations were performed under a laminar flow hood (class 100) with a relative humidity between 25 and 40% and a temperature of between 23 and 26 °C. The chip was placed on a stage having two adjacent Peltier elements. The temperature of the filling ports was dynamically kept 2–3 °C above the dew point to prevent water condensation across the ports. The dew point was calculated online from ambient temperature and relative humidity (HygroFlex1, Rotronic AG, Bassersdorf, Switzerland) and ranged from 1 to 10 °C. Typically, 2 to 3 aliquots of 300 nL of analyte molecules in either 1% BSA in PBS or DC culture medium were dispensed in the filling ports. The temperature of the capillary pumps was raised 5 to 20 °C above the dew point to adjust the flow rate through the microchannels between 20 and 100 nL min^{-1}. The flow rate of analyte solutions was set at $\sim$30 nL min^{-1}. The CSs were flushed four times with 300 nL of 1% BSA in PBS. The capillary pumps can be drained rapidly by briefly touching them with a wet tissue (cleanroom paper class 1, Contec, Spartanburg, SC). The PDMS substrate was removed, rinsed with 1% BSA in PBS (10 s), PBS (10 s), deionized water (2 s), and dried under N_2. The PDMS substrate was repositioned orthogonally on a second chip to bind the detection Abs to the captured analyte molecules. Subsequently, 600 nL of a solution of detection Ab was flown through the microchannels at a rate of 30 nL min^{-1}, and the CSs were rinsed and dried as described above.

The PDMS substrate was covered with a 0.17 mm thick microscope cover-glass (Karl Hecht, Sondheim, Germany) and the fluorescence signal was measured using a microarray scanner (GenePix Personal 4100, Axon Instruments Inc.) with a lateral resolution of 5 μm, and using an excitation wavelength of 635 nm and an emission band pass filter between 655 and 695 nm.

We varied the concentration of the capture Ab in the solution to coat the PDMS surface in order to optimize it for minimal consumption of Ab and maximum surface coverage. We found an optimum concentration of 100–200 μg mL^{-1} anti-TNF-α Ab to derivatize the PDMS surface with capture sites (data not shown).

2.5. Sample preparation

Dendritic cells were differentiated from peripheral blood mononuclear cells (PBMC) as described previously.[24] Briefly, monocytes were isolated from blood PBMCs by positive sorting with anti-CD14 magnetic beads (Miltenyi Biotech, Auburn, CA) and cultured in complete RPMI-1640 medium containing granulocyte macrophage-colony stimulating factor (GM-CSF, 50 ng mL^{-1}, Leucomax, Novartis, Basel, Switzerland) and interleukin-4 (3 ng mL^{-1}, R&D, Minneapolis, MN). Cell maturation was induced on day 5 by adding either lipopolysaccharide (LPS, 1 μg mL) from *E. coli* (Biowhittaker, Walkersville, Maryland) or recombinant human tumor necrosis factor (TNF-α, 10 ng mL^{-1}, R&D, Minneapolis, MN) as a means for TNF-α-induced TNF-α secretion. After incubation for 24 h, cell culture supernatant was obtained by centrifugation and directly assayed for TNF-α content. In order to obtain calibration curves, recombinant human TNF-α was diluted in DC culture medium.

2.6. Human TNF assay: a dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA)

TNF-α concentration in cell culture supernatant was determined by classical time-resolved fluorescence using EU-labelled streptavidin as a generic reagent to detect biotin-labeled Abs.[25,26] Briefly, a mouse anti-human TNF-α monoclonal Ab bound to a polystyrene 96-well plate (Wallac Oy, Turku, Finland) was used to capture the cytokine. After intensive washing with 0.05% Tween 20 in PBS, a corresponding biotinylated detection Ab was used to bind captured TNF-α and in a subsequent step, Eu-labeled streptavidin. Europium fluorescence was then measured with a time-resolved fluorometer (VICTOR 1420, Wallac/Perkin Elmer Life Sciences). In general, cell culture supernatant and TNF-α standard dilutions were measured in triplicates requiring a total of 150 μL per sample.

3. Results and discussion

3.1. Microfluidic system

The $\sim$2 cm^2 microfluidic chips used here are optimized to miniaturize solid phase immunoassays greatly while still being convenient to use, Fig. 2. The filling ports of each CS are $\sim$1 mm in size, wettable and surrounded by a non-wettable area. They can easily be filled by hand with 300 nL of solution using a micropipette or a low-volume pipetting robot. Each port is connected to a microchannel of a few millimeters in length, a width of 30 μm and a depth of 20 μm. Such a cross section generates a large capillary pressure relative to the filling ports. A shallow channel of 20 μm depth keeps the diffusion times of proteins from the bulk of the channel to the PDMS substrate within the range of a few seconds (*e.g.* $\sim$4 s for an

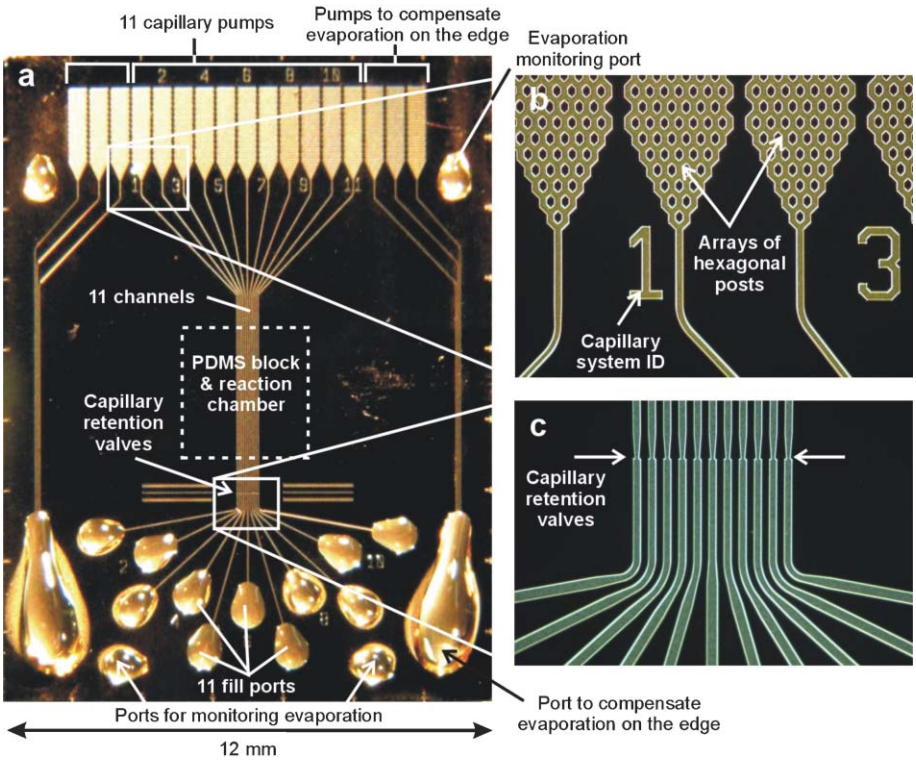

Fig. 2 Optical micrograph of a chip whose ports are filled with water. (a) This chip is a Si-microfabricated element covered with Au having eleven independent CSs. Each CS comprises a filling port (~ 1.5 mm wide, and 20 μm deep), a microchannel (with a 30 × 20 μm^2 cross section), a capillary retention valve, and a capillary pump. The reaction chamber forms when the PDMS substrate for the assay is placed over the parallel microchannels. Some additional ports compensate for the evaporation at the periphery of the chip or are used to monitor the evaporation. (b) Arrays of hexagonal structures generate the capillary force in the capillary pumps. (c) The capillary retention valves generate a strong capillary pressure and thereby retain liquid in the microchannels when the filling ports have been drained.

Ab). For the current experiments we mainly used microfluidic chips having 11 or 17 CSs. A 17 × 17 mosaic with 289 interaction sites has a footprint on the PDMS surface of only 1 × 1 mm^2 but contains as many interaction sites as three 96-well microtiter plates. The largest micromosaic shown in this work has 170 sites (see below).

The capillary pumps are created by structuring small, closely spaced wetting posts at the end of the CS, Fig. 2b. This geometry fulfills two purposes: providing a large volume for flushing enough material, a high capillary force ensured by the small dimensions and a small flow resistance due to the multiple parallel flow paths. This design also is particularly robust, because if one part is clogged or damaged, the areas placed behind it can still be filled with solution flowing in from the sides. Once filled, additional flow is induced by controlling the evaporation of liquid from the capillary pump; a filling port can therefore be drained entirely several times. However, the capillary retention valve in the microchannel at the beginning of the reaction zone maintains the channel underneath the PDMS block filled at all times, even if the fill port was emptied completely, Fig. 2c. The valve is the narrowest part of the CS; as the liquid passes through the narrow opening, the rear meniscus—which is initially in the filling port, and which is pulled into the access channel as the liquid is drained from the filling port and replaced by air–does not make it through the narrow opening. This is important because this retention valve provides the self-regulation mechanism that stops the flow when the filling port has been entirely drained.

Evaporation is controlled for all pumps in parallel using the Peltier element placed underneath the pumps, which has two advantages. First, it permitted us to design small capillary pumps that hold only a fraction of the assay volume. This is especially useful when assays require numerous solutions to be pipetted successively in the same filling port. Second, a small pump provides a small area for evaporation so that low flow rates can be achieved for the steps of the assay needing long incubation times. The collective control to all CSs can be applied so easily here because each CS is self-regulated, and because the long connection channels preclude the diffusion of waste material back from the capillary pump to underneath the PDMS slab. Each side of the chip has an additional CS composed of a "large" filling port, three microchannels and three capillary pumps. These CSs prevent the occurrence of different evaporation rates for the outer pumps of the array of CSs. In addition, two ports near the capillary pumps filled with water serve to monitor evaporation rates. Two ports next to the filling ports allow the proper dew point control to be observed in order to suppress the direct evaporation from the filling ports.

3.2. Surface immunofluorescence assay

We selected surface immunofluorescence as the format for the assay because it requires fewer steps than an ELISA, preserves the spatial localization of the signals, and many types of fluorophores can be employed as a label on detection Abs. Here, the excess of detection Ab is removed by flushing the CSs with buffers and water, after which the PDMS substrate is separated from the chip and placed in a fluorescence scanner.

Several factors can limit the sensitivity of a surface immunoassay, including the intrinsic binding characteristics of the capture and detection Abs (affinity and kinetics of binding, orientation on the surface), the non-specific deposition of analyte molecule and detection Ab on the surface, the binding of interfering molecules or the background fluorescence of the substrate.[18] We measured the background fluorescence of PDMS (see electronic supplementary information†) and thereafter selected fluorophores such as Cy5 or Alexa 647. Miniaturized immunoassays may also have specific

limitations owing to the depletion of reactants and the hydrodynamic flow properties of the system, which we discuss below.[5,27]

3.3. The risk of depletion of analyte molecules in miniaturized assays

The depletion of analyte molecules in miniaturized assays where both a small volume of sample is utilized and the analyte molecule is dilute may be an important limiting factor of the sensitivity of the assay.[27–29] In theory, depletion of analyte molecules could occur here *via* non-specific adsorption of TNF-α on the walls of the microfluidic chip and capture of TNF-α on the PDMS before the zone in which signal is measured. The non-specific adsorption was prevented by derivatizing the Au-coated microchannels with a protein-repellent monolayer of HS-PEG.[30] Since PDMS is homogeneously covered with capture Abs, there will be capture of TNF-α taking place as soon as the solution flows on the PDMS, which will not necessarily contribute to the generation of signal. This will be discussed next.

We take the example of 600 nL of a 250 pg mL^{-1} (*i.e.* 14.3 pM) solution of TNF-α to evaluate roughly the length on which the $\sim 5 \times 10^6$ TNF-α molecules (8.3×10^{-18} mol) contained in this sample might be captured. We estimate the footprint of a capture Ab on PDMS to be ~ 150 nm^2 in area[22,31] and suggest that 10% of them might have one antigen-binding region properly oriented to capture one TNF-α molecule from solution.[18] As the microchannels are 30 μm wide, there would be 20000 active capture sites every micrometer along the channel. Assuming an ideal case in which every active capture Ab molecule binds one TNF-α, the 5×10^6 TNF-α molecules from the sample would be captured on a 250 μm length of the microchannel. Monoclonal Abs obtained using classical immunization of mice usually have affinities in the range of $K_d = 10^{-9}$–10^{-11} M.[32] We can therefore expect that compared with the ideal case only a small fraction of the TNF-α present in the low-concentration analyte solution (14.3×10^{-12} M) will be captured. Taking 5% as a hypothetical fraction of surface-bound TNF-α, it would take ~ 5 mm to deplete TNF-α entirely from solution. This example shows that it is advantageous to employ narrow capture lines and, if possible, to limit the capture areas to the regions in which signals are read.[33] In practice, the flow rates, kinetics of capture, resolution of the signal-acquisition system, geometry of the capture areas, and coordinates of the signal on the surface all convolve and make it difficult to optimize the geometry of the capture sites using only theoretical models.[34,35] In the experiments presented below, the intersection between the lines on PDMS, where TNF-α is captured, and the microchannels, where detection Abs are provided, occurs ~ 1 mm after the beginning of the capture lines.

Fig. 3 shows a mosaic of fluorescence signals that reveal the capture of TNF-α on PDMS. The middle CS was filled with a 250 pg mL^{-1} solution of TNF-α and the other CSs with a 500 pg mL^{-1} solution. Identical concentrations of detection Abs were supplied along the horizontal direction. The columns of the mosaic exhibit a variation in the fluorescence signal of less than 10%, which indeed suggests that depletion of TNF-α along the channels was negligible, at least in this concentration range.

3.4. TNF-α micromosaic immunoassay

The sensitivity of the assay is investigated by filling adjacent CSs with solutions of TNF-α of diminishing concentration in a DC culture medium. The concentration of the detection Ab was varied as well to find the optimal dilution range. In addition, the concentration of TNF-α was determined in two

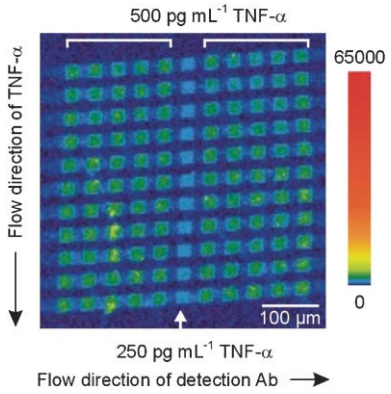

Fig. 3 Fluorescence image corresponding to the detection of TNF-α from solution using two chips with 11 CSs. TNF-α was captured by placing a PDMS block, whose surface was homogeneously covered with anti TNF-α capture Abs and blocked with BSA, on a first chip. Then the middle and the remaining 10 channels were filled with a 250 pg mL^{-1} and 500 pg mL^{-1} solution of TNF-α in PBS, respectively. After rinsing the channels, separating the PDMS block from the chip and drying it, the block was placed on a second chip orthogonal to the lines of captured TNF-α from the first patterning step direction. A 50 μg mL^{-1} solution of detection Ab labeled with Alexa-647 in PBS + 1% BSA was applied. The fluorescence signals are constant along the vertical direction, which indicates that depletion of TNF-α did not affect the assay for this range of analyte concentration. The sidebar indicates the (nonlinear) encoding of the fluorescence signal and is common to Fig. 4.

cell culture supernatants of DCs stimulated to produce TNF-α. The signals were recorded using a fluorescence scanner and are shown in Fig. 4. The signals are localized on the PDMS where the pairs of microchannels virtually crossed. There is also some fluorescence visible along the flow direction of the detection Abs in the part of the mosaic corresponding to high concentrations of TNF-α and detection Abs. We think that this fluorescence results from the detachment and re-capture of TNF-α molecules when solutions of detection Abs are flushed through the CSs. Two aliquots of 300 nL of TNF-α were supplied in each CS. The time for capture was set to 12 min for each aliquot by slowly evaporating the liquid from the capillary pumps. The flow rate in the channels was thus 25 nL min^{-1}.

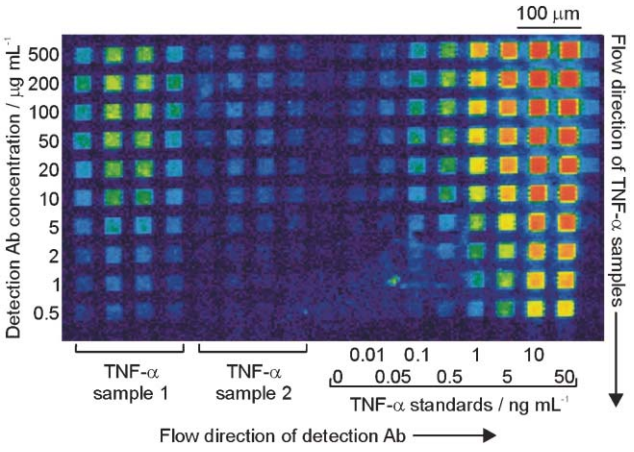

Fig. 4 Surface fluorescence immunoassay for TNF-α dissolved in standards and in cell culture supernatant solutions. The assay was characterized using DC culture media with known concentrations of TNF-α and solutions of detection Abs of varying concentrations. In addition, the concentration of TNF-α in two supernatant solutions of cultures of T-cells that were activated with two different factors was investigated. The detection Abs were labeled with Alexa 647 and the mosaic measured using a fluorescence scanner. The fluorescence signals suggest a sensitivity for this assay between 10 and 50 pg mL^{-1} when the concentration of detection Ab is $\geqslant 20$ μg mL^{-1}.

Indeed, short incubation times of less than 3–5 minutes for the capture of TNF-α compromised the sensitivity of the assay (data not shown). Fluorescence signals are visible for the 10 pg mL^{-1} solution of TNF-α when the concentration of detection Ab is $\geqslant$10 μg μL^{-1}. The graph in Fig. 5 represents the mean fluorescence values of each signal area as a function of the concentration of analyte molecules and detection Ab. The sensitivity for each concentration of detection Ab was calculated by defining the smallest concentration of TNF-α that had a mean signal above the mean background signal plus three times the standard deviation of the background.[18] The background is the signal for zero concentration analyte and corresponds to the middle column in Fig. 4. The sensitivity was found to be of the order of 20 pg mL^{-1} for the middle range of concentration of detection Ab ($\sim$20 μg mL^{-1}).

3.5. Comparison with DELFIA

We compare assays using the microfluidic chips with a state-of-the-art method such as DELFIA in Fig. 6. The DELFIAs were performed using 96-well microtiter plates and an incubation time of 2 h for the capture step. The assay required 6–7 h (excluding the deposition of capture Ab) and 150 μL samples. The amounts of TNF-α found in the supernatant of DC culture when the cells were stimulated using various factors are similar for both types of assay: the sensitivity of the microfluidic-based assay ($\sim$20 pg mL^{-1}) was slightly better than that of the DELFIA ($\sim$30 pg mL^{-1}). Here, the combinatorial aspect of the mosaic was mainly used to develop and characterize the assay for TNF-α, but the consumption of sample can even be made smaller by screening several types of analyte molecules in one sample using several types of capture Abs, as we have already demonstrated.[14,21] The microfluidic-based assay requires neither the addition of the enhancing solution to dissociate the europium reporter atom from the detection antibody nor sophisticated fluorescence equipment. Unlike assays carried out in microtiter wells, however, the number of truly independent assays is not equal to the number of sites forming the mosaic, but is limited to either the number of rows or columns of the mosaic.

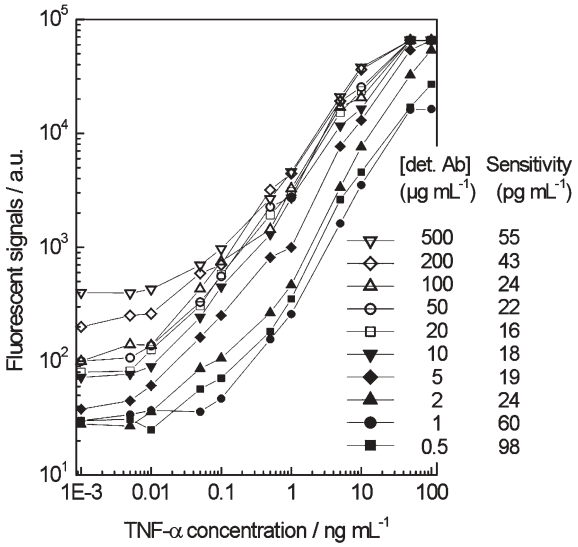

Fig. 5 Fluorescence signals corresponding to the detection of TNF-α using a solution of detection Ab with concentrations ranging from 0.5 to 500 μg mL^{-1}. These signals correspond to the part of the mosaic in Fig. 4 where solutions of TNF-α with a known concentration were provided. Too low a concentration of detection Ab does not lead to binding all the surface-captured analyte molecule, whereas at high concentrations the non-specific binding of detection Ab also limits the sensitivity. A concentration range of detection Ab between 2 and 100 μg mL^{-1} yields a maximal sensitivity of $\sim$20 pg mL^{-1}.

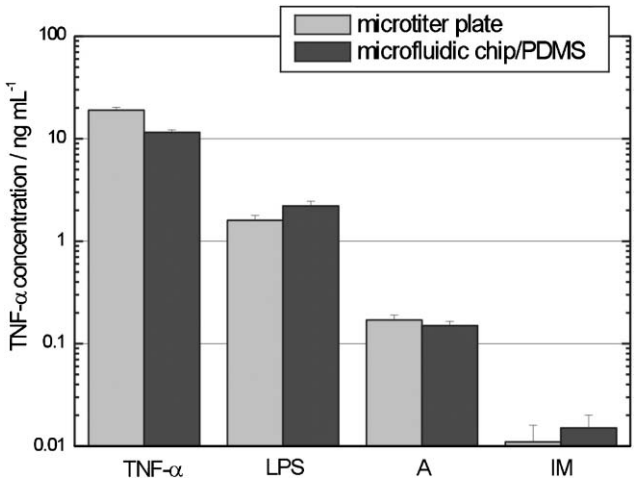

Fig. 6 Comparison between DELFIAs using microtiter plates and microfluidic chips using PDMS substrates for detecting TNF-α in various DC culture supernatants. The supernatants are categorized based on the factors used to differentiate the DCs. DELFIAs required 150 μL of supernatant and took 6–7 h while the microfluidic-based assays worked with 600 nL of supernatant and were performed in less than one hour.

4. Concluding remarks

The main achievement presented in this work, which is the detection of an analyte molecule in very small sample volumes with $\sim$1 pM sensitivity, was realized by optimizing many aspects of the assay. The design of the microfluidic chips and the use of Peltier elements permit the evaporation in the filling ports and in the capillary pumps to be compensated and monitored. This helps to control the flow of liquids in the channels and hence to optimize the duration of the incubation steps. The detection method also plays an important role. It is desirable to employ long-wavelength fluorescent tags to avoid background fluorescence from the PDMS surface. The fluorescence signals were read using a biochip scanner. We are confident that a higher sensitivity can be achieved using, for example, a fluorescence microscope or other types of detection platforms, polyclonal detection Abs, a different immobilization procedure of the capture Abs on the PDMS surface, or fluorescent particles such as nanoparticles. We note that the miniaturized assay shown here has a comparable sensitivity to a DELFIA performed using microtiter plates. It is clearly advantageous to employ microfluidics for such assays because they are highly sensitive and require only minute amounts of cell culture supernatant. We are therefore confident that the method presented here is suited for the rapid development of immunoassays and for performing multiple tests in parallel to detect for example cytokines or chemokines with very high sensitivity and economy of sample. Hence, this assay also opens the possibility to analyze serum or plasma samples derived from mice or rats which are the focus of toxicological studies in drug development. Such analysis was, as yet, not feasible due to limitations with classical ELISA-type assays involving at least 100-fold larger sample volumes.

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