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Microfluidics for Processing Surfaces and Miniaturizing Biological Assays**

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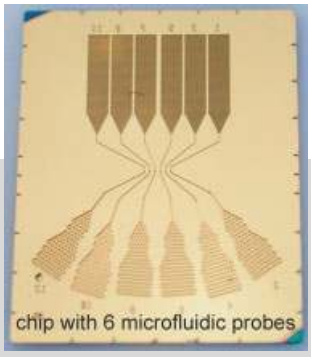

This review is an account of our efforts to develop a versatile and flexible microfluidic technology for surface-processing applications and miniaturizing biological assays. The review is presented in the context of current trends in microfluidic technology and addresses some of the major challenges for confining chemical and biochemical processes on surfaces: the sealing of a microchannel with a surface, the world-to-chip interface, the displacement of liquids in small conduits, the sequential delivery of multiple solutions, the accurate patterning of surfaces, the coincident detection of various analytes, and the detection of analytes in a small and dilute sample. Our solutions to these problems include the use of reversible sealing, capillary phenomena for powering and controlling liquid transport, and non-contact microfluidics for spotting and drawing (on surfaces) with flow conditions. These solutions offer many advantages over conventional techniques for handling minute amounts of liquids and may find applications in lithography, biopatterning (e.g., the patterning of biomolecules), diagnostics, drug discovery, and also cellular assays.

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1. Introduction to Microfluidic Systems

Microfluidics refers to the handling of liquids or gases at a scale generally below 1 mm, where a number of phenomena that are not present or not predominant at larger scales can be exploited for numerous purposes.[1] The field of microfluidics is in essence multidisciplinary as it combines microfabrication techniques with chemistry and biology.[2,3] Within microfluidics, the currencies are nanoliters for reaction volumes,[4] micrometers for dimensions,[5] and milliseconds for diffusion and reaction times.[6,7] The accurate sampling, positioning, and transport of nanoliter volumes of liquids using microfluidics mirror the high dimensional control with which microfluidic devices are made. In this review, we describe a technology based on microfluidic networks (MFNs) and microfluidic probes (MFPs) that is useful to localize chemical and biochemical reactions on a surface, where the possibility of reversibly placing a microfluidic element in contact with or close to a surface constitutes an essential feature for attaining patterning resolutions on the submicrometer scale.[5,8]

Microfluidics have characteristics that are sometimes counterintuitive to chemists and biologists, who are used to working with Erlenmeyer flasks and microtiter plates: at the micrometer scale, surface tension and capillary effects are important, and "advanced" pumping and liquid-handling methods become necessary to overcome the hydraulic resistance of micrometer-sized conduits. Similarly, the flow of liquids in microchannels is laminar, and therefore mixing at the micrometer scale can, in fact, be difficult to achieve.[9–14] The large surface-to-volume ratios of microchannels can make interfacial reactions efficient[15–17] but this also entails the risk of depletion of reactants.[8] Microfluidic devices sometimes comprise a rich mix of elements and functionalities,[18] making them efficient for tasks as elementary as metering liquids as well as for processing and analyzing complex biological specimens.[19–21] In fact, they can be as simple as glass capillary tubes[22] or as complex as chips having micromechanical pumps[23,24] and electronic circuitry to move liquids[25] or even suspended cells that use, for example, electro-osmotic flow,[26,27] dielectrophoretic pumping,[28,29] or electrowetting.[30–34] Figure 1 shows a number of applications that take advantage of the phenomena typical of microfluidic devices. These applications can roughly be divided into two categories. Firstly, microfluidics enables patterning/processing of surfaces with chemicals,[35,36] polymers,[37–40] beads,[41,42] biomolecules,[5] and even cells,[43–45] which cannot be easily achieved using conventional lithographic methods.[46] Protein chips, for instance, are most often prepared using inkjet-, pin-, or quill-spotting techniques.[47,48] These applications also comprise the directed assembly of elements into prototype devices or func-

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Heinz Schmid joined the IBM Zürich Research Laboratory in 1984, where he is employed as Senior Engineer in the Science and Technology Department. After finishing his physics apprenticeship diploma in 1988, he worked on electron and ion field-emission sources and low-energy electron holography. He later moved on to work on emerging lithographic techniques, including imprinting, soft lithography, and, in particular, microcontact printing. In addition, his recent activities include the development of microfluidic systems for patterning surfaces. His current field of interest concerns the synthesis and characterization of semiconducting nanowires.

David Juncker received a degree in electronics–physics from the Institute of Microtechnology, University of Neuchâtel, Switzerland, in 1996. Shortly thereafter, he was awarded a one-year fellowship from the Swiss Academy of Engineering Sciences for research on a microelectromechanical (MEMS) accelerometer at the Metrology Institute of Japan in Tsukuba from 1997–1998. From 1999 to 2004, he worked at the IBM Zürich Research Laboratory on soft lithography and microfluidics for high-resolution surface patterning, ultraminiaturized diagnostics, and single-cell processing. During this time, he earned his Ph.D. (2002, University of Neuchâtel). His current interests are in the development of novel nanotechnologies and microfluidic systems for use in biology and medicine.

ADVANCED MATERIALS

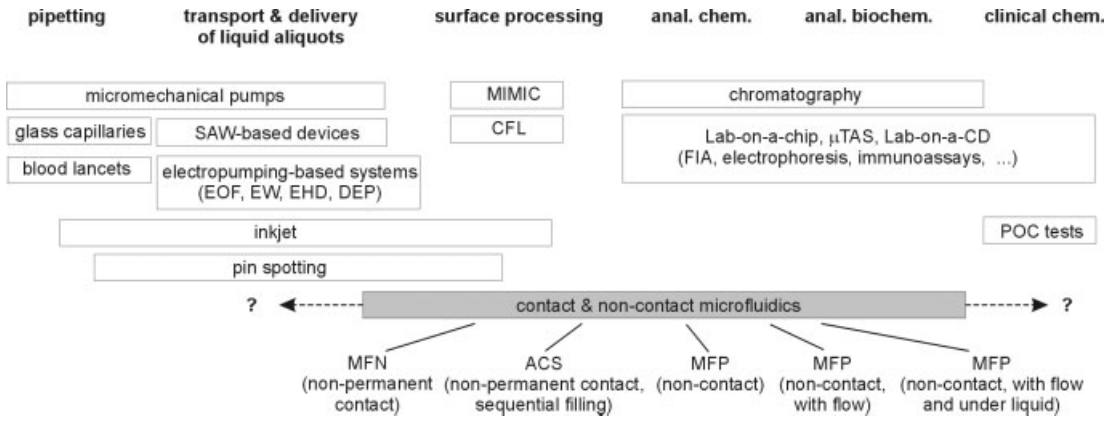

Figure 1. Microfluidics utilizes many principles to move, process, and analyze small volumes of solution and, therefore, cover a wide range of applications, some of which are shown here. Here, SAW refers to surface acoustic wave, EOF to electro-osmosis flow, EW to electrowetting, EHD to electrohydrodynamics, DEP to dielectrophoretic pumping, MIMIC to micromolding in capillaries, CFL to capillary force lithography, μTAS to micro-total analysis systems, FIA to flow-injection assays, POC to point-of-care, and ACS to autonomous capillary system.

tional structures.[49–55] Secondly, the seminal work by Manz and co-workers on chip-based capillary electrophoresis[56,57] helped many concepts, such as lab-on-a-chip or micro-total analysis systems,[58–64] micro-flow injection analysis,[65] lab-on-a-CD,[66–68] and microfluidic processors,[69] to emerge and find applications in analytical chemistry and biochemistry, diagnostics,[70] and research in life sciences.[71–73] These concepts come with various exciting flavors, such as parallelism,[74,75] fast time to result,[76–80] portability,[81–83] high sensitivity,[84] small volume of sample,[85] high-quality data,[57,86,87] heterogeneous phase reactions with large surface-to-volume ratios,[16] efficient particle/cell sorting,[88–90] or reactions done at liquid interphases.[91–94] Microfluidic systems for crystallizing proteins illustrate well how several of the above features can be combined for one application.[95–98]

The heart of a microfluidic device is often a microchannel, in which reaction, separation, or detection takes place. For this reason, thorough work has been done on optimizing the geometry of microchannels to improve their hydrodynamic properties or separation performances.[99–102] Microfluidic systems having long microchannels (up to several centimeters) have also been devised to synthesize colloidal particles[103] or to align very long DNA molecules on surfaces.[104] Recent developments have brought the interesting possibilities of moving and mixing liquids using acoustic waves[105] or using the instability of liquids on surfaces having wetting patterns.[106] Early microfluidic chips were mostly fabricated in silicon, glass, or quartz and were typically sealed.[18,71,73,107–110] Many more materials (most notably plastics) are now being used,[111–116] and there is a trend to developing research devices in silicon or poly(dimethylsiloxane) (PDMS) by using conventional microfabrication techniques, rapid prototyping, or soft lithography,[5,86,117–119] before eventually mass-fabricating successful prototypes using lower-cost polymeric materials and higher-throughput techniques.

The use of microfluidics for surface patterning and miniaturized diagnostics is desirable because of the economy of reagents and the potential for parallelization. Yet, many parallelized operations only partially include microfluidics despite the many advantages described above. For example, high-throughput screening for drug discovery is generally still done using the wells of microtiter plates, and microarrays are typically patterned using mere pins. Among the reasons that prevent more widespread adoption of microfluidics are the issues of having i) large dead volume, ii) awkward chip-to-world interfaces, iii) difficulties in exchanging solutions sequentially, iv) limited parallelization and throughput due to bulky interfaces and complex flow-control systems, and v) the difficulty of processing large volumes or areas. Notable counter-examples, which are also commercial successes, include the microfluidic high-throughput screening station from Caliper Life Sciences, which uses a small sipper capillary to sample aliquots from standard microtiter wells and a combination of pressure and electrokinetic pumping to perform analyses.[78,120] High throughput is achieved here by miniaturizing the volume for the assay, thereby making the assay shorter, by rapid sequential pipetting without dead volume, and by parallelization using as many as twelve channels. Another example is the crystallization of proteins, where microfluidics are used to examine the optimal conditions to crystallize proteins in a combinatorial manner. In this case, dead volumes, the sequential exchange of solutions, and the chip-to-world interfaces are secondary because only a few different solutions of proteins need to be filled only once on the microfluidic chip.

Here, we review microfluidic networks,[5] their subsequent developments, and new types of contact-free microfluidic systems called microfluidic probes. MFNs are a subset of microfluidic systems characterized by arrays of microchannels and by the use of capillary phenomena to fill and flush these microchannels. This approach and variants thereof try to solve many issues plaguing microfluidic systems by suppressing dead volumes, offering intuitive interfaces that can be serviced using standard micropipettes, enabling the sequential filling of solutions, and supporting parallelization by arraying

programmed functional units. Finally, the more recent development of MFPs allows the processing of large areas with microfluidic streams. We will show how these techniques can be applied to a wide range of applications by providing reactants and liquids locally to a surface under "microfluidic conditions" (Fig. 1). We will briefly describe how MFNs can be used to guide liquids on surfaces,[8] pattern proteins, and perform combinatorial surface immunoassays.[121] Then, we will describe how to use capillary phenomena to implement various functions into MFNs. Such networks are called autonomous capillary systems (ACSs) because they are programmed to fulfill a function and are self-powered; they can pattern proteins, in particular on hydrophobic elastomeric substrates, with great accuracy, and effect surface immunoassays.[122] MFPs operate in non-contact mode, i.e., a ~10 μm gap separates the microfluidic chip and the substrate, and can operate in air or in liquid. MFPs working in air rely on the formation of a capillary bridge between some of their microstructures and the surface.[123] In the simplest case, a "static" capillary bridge forms inside the gap between the MFP and the surface, and the substrate surface can thus be processed by drawing the MFP over the surface. A more interesting possibility is an MFP with automatic operation that is powered and controlled by capillary phenomena: when the probe is approached sufficiently close to a substrate, a solution is automatically drawn into the gap and flushed across the surface until the MFP is retracted, thereby abruptly interrupting the laminar flow. Finally, a variant of the MFP operates immersed in liquid and exploits hydrodynamic effects for confining a microjet that can localize additive and subtractive processes on a surface also immersed in the liquid.[124]

2. Microfluidics for Surface Processing

Surface processing can greatly benefit from microfluidics because many emerging applications require surfaces to be decorated with inks and molecules that are not compatible with the typical conditions encountered in conventional photolithography. Photolithography involves UV light, organic solvents, steps done at elevated temperatures, low- and high-pH baths to develop exposed photo- or electron-beam resists, and aggressive etch baths,[125] each of which can degrade biomolecules, organic molecules, and sometimes even polymers. The emergence of organic-based circuits (polymer electronics, organic light-emitting diodes) and unusual (e.g., flexible or curved) substrates calls for alternative patterning methods, in which polymeric inks and viscous liquids can be delivered locally on a surface.[126–130] In biology, protein chips require the spatially controlled deposition of proteins on appropriately derivatized substrates, and sensor elements must be functionalized with biochemical receptors for specific and sensitive biosensing of analytes.[47,131] Proteins and biomolecules are best patterned on surfaces by deposition from a small volume of water or buffer solution. Techniques for patterning proteins using microfluidics can essentially be divided into two categories. The first is based on non-contact methods, where nanoliter or smaller volumes of protein solution are spotted onto a surface. These techniques employ inkjet technology or other advanced microfluidic devices.[47,132] The second category requires a gentle contact between a pin, or a capillary, with a surface to transfer a small volume of protein solution onto the surface.[47,48] Both contact and non-contact techniques are attractive for their versatility, high throughput, and ability to pattern large numbers of different types of protein. Their drawback is that the quality of the spots is affected by uncontrolled wetting and subsequent evaporation.[133–135] We are interested in developing microfluidic-based patterning methods that bear the intrinsic advantages of microfluidics, overcoming the above disadvantage, and that approach the accuracy and precision found in lithography while having the ability to perform sequential processes.

2.1. Sealing a Microfluidic Device with a Surface

The costs associated with photolithography, which necessitates expensive clean rooms, lithographic and metallization tools, as well as the use of relatively large quantities of chemicals, are circumvented in soft lithography by using a structured PDMS elastomer as the patterning vehicle.[136] PDMS can easily be structured by pouring it as a liquid on a mold, curing it, and detaching it from the mold. A single microfabricated master mold can thus be replicated multiple times with ease. PDMS is non-toxic (once cured), commercially available and cheap, hydrophobic, optically transparent, permeable to air but not to water, and chemically inert.[137] Soft lithography has flourished into an impressive number of variants to provide a broad scope of patterning techniques and research platforms. The essential attribute of soft lithography is the spontaneous, intimate contact formed between reasonably smooth surfaces and the soft PDMS owing to the work of adhesion between the contacted surface and PDMS.[138] This contact occurs on the molecular scale, is termed conformal, and is "gentle" and fully reversible; a stamp placed on a surface can easily be peeled off anytime. The placement of a surface-patterned PDMS onto a surface therefore leads to a contact between the raised PDMS parts and the surface with submicrometer lateral accuracy. This property has been exploited in microcontact printing, where structured stamps are used to pattern for example monolayer-forming molecules,[139] polymers,[140–143] metallic films,[144,145] proteins,[146] and catalysts[147,148] on various surfaces.

Structured PDMS stamps can also be used as microfluidic vehicles by first placing them in contact with smooth surfaces and then filling liquids into the cavities engraved in the PDMS. Alternatively, flat PDMS stamps can be used as substrates and sealed with MFNs structured into hard materials, such as Si or glass.[8] We have explored all combinations of hard or soft MFNs and substrates. MFNs in PDMS are easy to fabricate unless the aspect ratio (width-to-depth ratio) of the microchannels and other small structures is too high. Deep and narrow elastomeric structures are prone to collapse and subject to de-

formation during release from the mold and when there are used.[149,150] Large reservoirs in a PDMS layer may collapse on a surface as well. In the latter case, adding stabilizing posts is a simple remedy to prevent the collapse of large, unsupported structures,[8] or the formulation of PDMS can be varied to make it harder.[151] MFNs in PDMS could in principle be used to pattern proteins on glass substrates, Si wafers, plastics, metals, and various sensing surfaces (chips for surface-plasmon resonance, grating couplers, quartz-crystal microbalances, electrodes, etc.).[152,153] Conversely, MFNs made from hard materials, such as Si, glass, or plastics, can be used to pattern PDMS surfaces. The adsorption of protein from solution to PDMS is, like on many other hydrophobic surfaces, rapid, spontaneous, and largely irreversible,[154–156] which alleviates the need to treat PDMS surfaces for binding protein molecules.[157] Two hard surfaces in contact may not conform and have asperities that can cause a liquid to leak outside the region of a surface where confinement is sought. In this case, strategies to prevent the liquid from leaking into areas not targeted for patterning are needed and will be discussed below.

2.2. Transporting Liquids in Microchannels

Transporting liquids at the micrometer scale can be done using active pumping or "passive" forces. The flow rate of a liquid filling a microchannel can be expressed in a form similar to the relation between electrical current, resistance, and potential:

$$Q = \frac{1}{\eta}\frac{\Delta P}{R_F} \quad (1)$$

where η is the viscosity of the liquid, ΔP is the difference in pressure inside and in front of the liquid, and R_F is the resistance to flow of the microchannel. In many systems, ΔP is typically created by a pump or rotating device.[18] Capillary forces provide an alternative means to create a pressure difference in small conduits. In most lab-on-a-chip applications, the microchannels have a rectangular cross section, and the resulting capillary pressure P_c of a liquid–air meniscus in such a microchannel is

$$P_c = -\gamma\left(\frac{\cos a_b + \cos a_t}{d} + \frac{\cos a_l + \cos a_r}{w}\right) \quad (2)$$

where γ is the surface tension of the liquid, $a_{b,t,l,r}$ are the contact angles of the liquid on the bottom, top, left, and right walls, respectively, and d and w are the depth and width of the microchannel, respectively. Working with capillary forces removes the need of having to use peripherals.[8,37,38,122] Each wettable ($a < 90°$) wall of the microchannel contributes to generating a negative pressure in front of the liquid and to drawing the liquid into the channel. Figure 2A illustrates the filling due to capillary pressure of a microchannel sealing a surface.

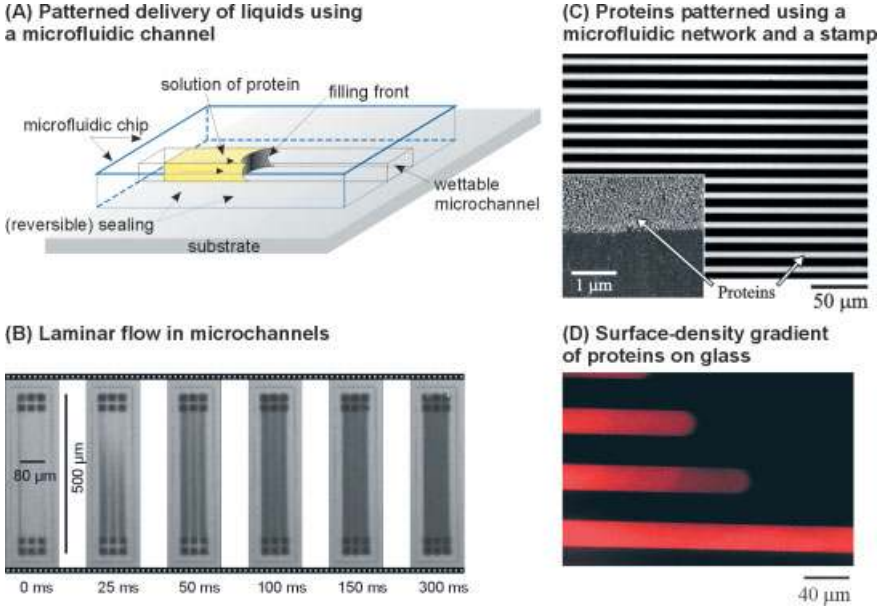

Figure 2. Surface processing using MFNs and related diffusion phenomena, patterning resolution, and depletion of reactants. A) MFNs are formed by sealing a surface with a microfabricated structure, wherein the surface or the structure is typically made from PDMS. Sealing ensures that solutions filling the microchannels due to capillary pressure remain well confined to the channels, thereby allowing high-resolution processing of the substrate. B) Optical micrographs showing the displacement of water in a microchannel by water containing a black colorant that enters the microchannel through six vias connected to a single channel. The black stripes separated by water reflect the position of the vias and illustrate the laminar nature (no turbulent mixing) of the flow. C) Fluorescence micrograph of fluorescently labeled antibodies (Abs) that were deposited onto a hydrophobic PDMS layer and subsequently printed onto a glass surface. Transfer to the glass made it possible to visualize the resolution and contrast of the patterns of protein using atomic force microscopy (AFM). The AFM micrograph (inset) reveals that the deposition of the antibody protein molecules occurred with a resolution of ~100 nm. D) Fluorescence micrograph showing short-scale gradients of TRITC-labeled (TRITC: tetramethyl rhodamine isothiocyanate) Abs that were deposited using an MFN in PDMS onto a glass surface. (Adapted with permission from [175,178]. Copyright 2000, American Chemical Society.)

The exact solution to the flow-rate resistance of such microchannels, which has been adapted from the literature,[158] is a geometric term with a Fourier series:

$$R_F = \left[\frac{ab}{12}\frac{a^2}{L}\left(1 - 3\frac{a}{b}\frac{64}{5\pi^5}\right)\frac{\sum_{n=1}^{\infty}\tanh\left(m(n,a)\right)b/2}{(2n-1)^5}\right]^{-1} \quad (3)$$

where a and b are the width or depth, respectively, satisfying the condition $a < b$. L is the length of the microchannel, and m(n,a) = (π/a)(2n–1). Equation 3 can be approximated by a linear solution

$$R_F = \left[\frac{1}{12}\frac{(a+b)^2}{b^2}\left(1 - \frac{1}{8}\frac{a}{b}\right)\frac{abR_H^2}{L}\right]^{-1} \quad (4)$$

where R_H is the hydraulic radius of the microchannel $R_H = 2P/A = ab/(a+b)$, with P being the perimeter and A the area of the cross section of the microchannel.

The flow in a microchannel can thus be calculated, and, in the case of capillary filling, spontaneous filling of a liquid inside a microstructure results from the interplay between the surface tension of the liquid and the chemistry and geometry of the surfaces of the microstructure,[159] divided by the flow-rate resistance that continually increases as the channel is being filled by the flow.[8,122]

Applying positive pressures to force a liquid into a microfluidic channel may overcome the work of adhesion between a PDMS layer and a hard surface. This would likely be a problem for small channels, for which the flow resistance can be high because it scales with the fourth power of the inverse of the channel cross section. If one considers the average velocity of flow, the scaling is still to the second power. Microfluidics powered by capillary forces is advantageous (Fig. 2A) because capillary forces i) generate negative pressures and thus assist sealing and ii) scale favorably. Capillary forces increase with the inverse of the smallest dimension and thus can partially compensate for the increase of flow resistance in contrast to many other pumping schemes.

Adjusting the capillary pressure by changing the wettability of one wall only, namely, the substrate ($\cos\alpha_b$), is generally not practical. Changing the wettability of the three microchannel walls is a more efficient way to adjust the capillary pressure and can be done with a large variety of chemical processes.[160–162] MFNs in Si/SiO_2 and glass can be silanized[163,164] or covered with sputtered Au, for example. One advantage of using Au-covered MFNs is the possibility to program the wettability and protein repellency of the Au surfaces accurately using thiols with different end groups by forming self-assembled monolayers.[165,166] It is comparably more difficult to make MFNs hydrophilic in PDMS. The hydrophilization of PDMS requires strong oxidative treatments (e.g., exposure to strong oxidants, such as aqueous permanganate, UV-ozone or O_2-based plasma),[138,167–169] and PDMS generally recovers its original hydrophobicity within a few minutes owing to the migration of low-molecular-weight siloxane chains from the bulk to the surface.[170–172] Storing PDMS under water after the hydrophilization treatment or grafting hydrophilic polymers slows the hydrophobic recovery process.[173,174]

An important characteristic of microfluidics is the laminar flow condition. A measure of the laminar or turbulent properties of liquid flow is the Reynolds number Re:

$$Re = \frac{2R_H\rho v}{\eta} \quad (5)$$

where ρ is the liquid density and v its flow velocity. This number expresses the ratio of inertial forces to viscous forces. Reynolds empirically established a limit of 2300, above which flows become turbulent and below which they are laminar. The example shown in Figure 2B, where water in a microchannel with a 6 μm × 80 μm cross section is displaced by a colored solution flowing at a velocity of ~1.5 cm s^{-1} yields Re = 1.7. The colored solution enters the microchannels as six streams through six out-of-plane vias that are located at the bottom of the microchannels. The depth of such a microchannel is 6 μm, whereas the walls separating the vias are ~12 μm wide. The low aspect ratio and the laminar flow in the microchannels prevent the incoming streams from displacing the liquid positioned underneath (downstream) of the separation walls. It takes ~300 ms before all of the transparent solution is exchanged by lateral diffusion.

2.3. Patterning of Surfaces Using Microfluidics

The ability of MFNs to pattern a surface locally with high resolution and accuracy is illustrated in Figure 2C. There, antibody molecules labeled with TRITC fluorophores were patterned as lines, 5 μm wide, on a hydrophobic PDMS surface using a Si-microfabricated MFN.[175] The patterned proteins were then microcontact printed onto a glass slide to allow the patterns to be imaged at high resolution using atomic force microscopy (AFM). The AFM micrograph obtained at the edge of one line of proteins has a resolution on the order of 100 nm, and no protein seems to be present outside the zones that were exposed to the interior of the microchannels. The passive adsorption of proteins on hydrophobic surfaces is a fast process that is limited at macroscopic scales by diffusion. Here, the deposition was completed within 2 min using a 200 μg mL^{-1} solution of antibody in phosphate-buffered saline (PBS).

The smallest dimension of a microchannel, which is usually the depth because it is convenient to microfabricate wide and shallow structures, plays a dominant role in terms of the hydrodynamic and kinetic behaviors found in microfluidic systems. The characteristic time t for diffusion along one dimension x is proportional to x^2/D, where D, the diffusion coefficient of the molecule in a liquid, can be estimated using the Stokes–Einstein relation

$$D = kT/(6\pi\eta r_h) \quad (6)$$

Here, r_h is the hydrodynamic radius of the molecule, η the viscosity of the liquid, T the temperature, and k the Boltzmann

constant.[176] For example, a protein that has a molecular weight of 20 kDa (1 Da = 1.66×10^{-27} kg) and one hydration layer has a diffusion constant $D \sim 10^{-10}$ m^2 s^{-1} at ambient temperature.[1,177] Such a protein has a characteristic time for diffusion along a length of 1 μm of only ~10 ms. Under static conditions (no flow), the linear diffusion equation derived from Ficks first law,

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial^2 t} \tag{7}$$

gives the flux of proteins (or reactant in general) reaching a substrate exposed in a microchannel initially filled with a solution of protein of defined initial concentration. We showed in previous work that a complete monolayer of antibody can be deposited on PDMS in 10 ms from a 1 mg mL^{-1} solution of antibody in PBS using 1.2 μm deep microchannels.[8] It is important to note that in the case of surface processing using reactants in microchannels, depletion of reactant is quickly achieved unless more reagent is supplied via convection (flow).

Depletion effects occurring in microfluidics in fact provide an efficient route to form surface-density gradients of proteins on surfaces on length scales that are not yet accessible using other methods (Fig. 2D).[8,178–181] Interestingly, the length scales and diffusion times found in microchannels compare with biochemical processes occurring in living cells.[19,182,183]

The ratio between convective and diffusive transport is measured by the (dimensionless) Péclet number:

$$Pe = \frac{2R_H v}{D} \tag{8}$$

This number is helpful in designing microfluidic structures where the diffusion of reactants should be maximum, such as for surface immunoassays and surface processing, or limited, as it should occur with separation systems.[184] The interplay between laminar flow, convection, and diffusion provides exciting strategies for bioanalysis,[185,186] patterning and addressing supported lipid bilayers,[187] and manipulating plugs of liquids in multiphase systems.[188,189]

3. Surface Assays

There are many formats for surface assays depending on i) the nature of the surface-immobilized receptor, ii) the assay design, and iii) the type of signal employed to report the binding between a receptor and an analyte.[190–192] Immunoassays involve antibodies and antigens as binding partners and form an important class of biological assays, as they can detect small molecules, peptides, proteins, infectious agents, pollutants, pesticides, cells, and hormones in complex samples and with high sensitivity.[193–195] They are used for research in life sciences, prognostics and diagnostics, therapy monitoring, environmental control, and quality control in the pharmaceutical and biotechnological industries. Techniques to produce and engineer antibodies for specific antigens are numerous. A common step for surface immunoassays is to employ a surface-immobilized antibody (antigen) to bind specifically an antigen (antibody) analyte from solution. This step is termed "capture". A majority of surface immunoassays uses a detection antibody to bind captured analytes. The detection antibody is typically conjugated with an enzyme, fluorescent dye, or (nano)particle as a means to generate an optical or electrical signal that is proportional to the concentration of analyte in the sample.[193]

Fluorescence is a signal format that reinforces the benefits of miniaturization: fluorescence microscopes and scanners along with solid-state lasers, charge-coupled devices, photo-multiplier tubes, and fluorescent-dye chemistry have seen dramatic progress over the past few years. It is now easy and convenient to use a fluorescence scanner to measure fluorescence signals on a surface with a lateral resolution of a few micrometers and a detection sensitivity of < 1 dye μm^{-2}.[196] New dyes with long wavelengths of excitation and emission are being developed to lower fluorescence background from biological specimens.[197] It can similarly be advantageous to employ "quantum-dot" dyes, which are less prone to photobleaching and have relatively well-defined fluorescent properties compared with their organic counterparts.[198,199] Moreover, fluorescence immunoassays require fewer steps than is required assays using detection antibodies conjugated to enzymes.

3.1. Miniaturized and Combinatorial Surface Assays

The combination of the above-mentioned developments and the accurate patterning of proteins on surfaces using MFNs open the exciting possibility of miniaturizing surface assays with the attendant benefit of using small volumes of samples and reagents, screening for multiple analytes in parallel, and making assays fast. Using an MFN having a series of independent microchannels allows many parameters to be varied in a single experiment. Different samples can be measured in parallel, and, even better, combinatorial assays can be done using two MFNs: the first MFN can pattern lines of different receptors (capture Abs) on a substrate, and the second MFN provides a series of samples across the lines of capture Abs.[121,200] A proof-of-concept for such an assay, which we call micromosaic immunoassay, is shown in Figure 3A. Here, the first series of microchannels were all filled with a solution containing TRITC-labeled rabbit antibodies. The rabbit antibodies adsorbed on the PDMS surface. Next, the entire PDMS surface was exposed to a solution of bovine serum albumin (BSA) to prevent non-specific deposition of proteins during the next step. A similar MFN was used to supply anti-rabbit antibodies in a perpendicular direction. In this proof-of-concept, the surface-immobilized rabbit antibodies acted as antigens for anti-rabbit antibodies, and binding occurred where the channels "crossed" on the surface.

Figure 3B extends the proof-of-concept to the simultaneous detection of anti-species antibodies from solutions. Using n and m microchannels for a micromosaic immunoassay yields $n \times m$ binding sites but only requires $n + m$ pipetting steps.[74] A small volume of sample, typically less than 1 μL, is used to

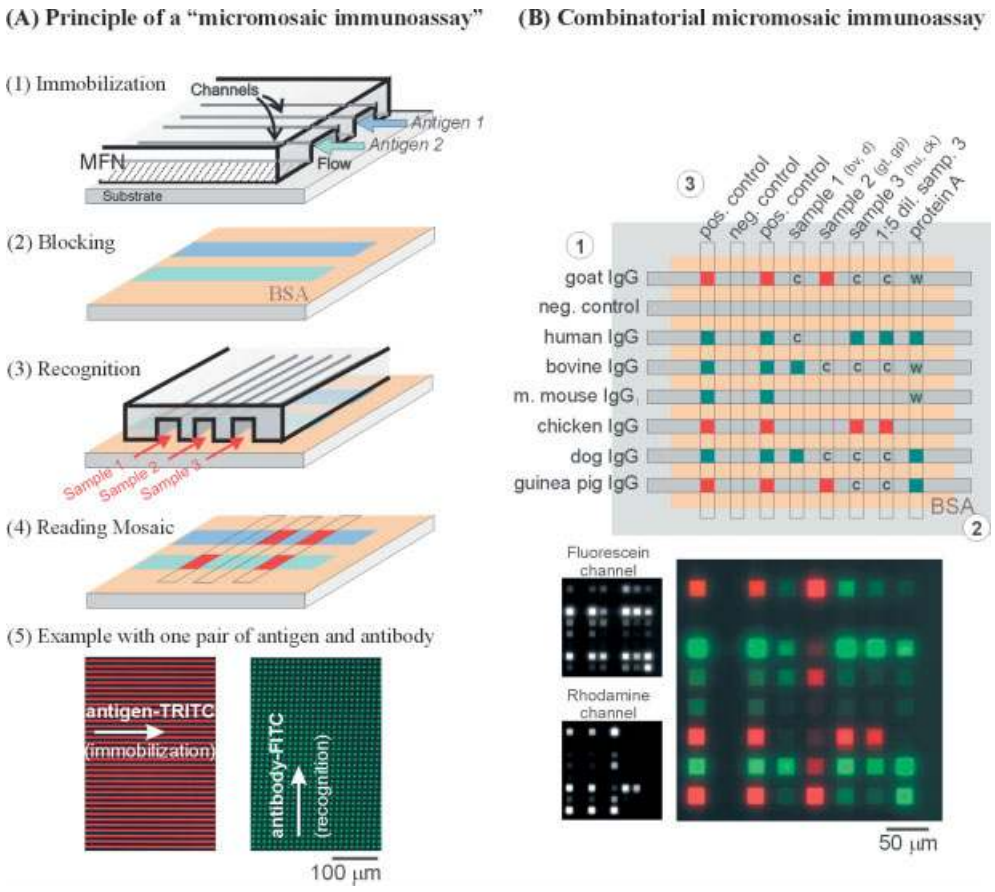

Figure 3. Micromosaic immunoassay. The principle of such an assay is to localize surface-binding events taking place in the independent microchannels of an MFN. A) A simple variant is to deposit various lines of antigen molecules on PDMS using an MFN. After removal of the MFN, the substrate for the assay is rinsed and exposed to bovine serum albumin (BSA) from solution to prevent non-specific deposition of proteins in the later steps. A second MFN, rotated by 90° to the first one, delivers a series of samples across the lines of surface-immobilized antigens. Antibodies from the sample may bind their specific surface-immobilized antigens. In the fluorescence micrographs, lines of TRITC-labeled antigens were deposited on a PDMS surface, which bound FITC (fluorescein isothiocyanate) antibodies from solution during the recognition step. For demonstration purposes, the microchannels shown here were not independent. B) Two MFNs, having eight independent microchannels 10 μm wide, were used to interrogate the binding between surface-immobilized receptors (protein A, or antibodies from various organisms) with fluorescently labeled detection antibodies. This micromosaic immunoassay was done in less than 20 min. (Adapted with permission from [121]. Copyright 2000, American Chemical Society.)

screen for *m* potential binding events, and the level of integration reached with this method is high considering that 64 test sites occupy only 0.16 mm^2 of a PDMS surface. A micromosaic immunoassay experiment, such as the one shown in Figure 3B, necessitates ~10 times less time, ~100 times less volume of samples and reagents, and ~1000 times less area per site than an assay done using a 1536-well microtiter plate. An important difference between the micromosaic immunoassay and the microtiter plate format is, however, that the binding sites of the mosaic are not all independent: cross-reactivity might occur as one solution runs across multiple binding sites on a surface.[121] Interestingly, new methods are now being employed in which the bottom of wells of microtiter plates are derivatized with a microarray of capture sites for the simultaneous screening of analytes,[201,202] or in which the reaction sites for detection are scaled down.[203–205]

Micromosaic immunoassays were recently used to detect cardiac markers from human plasma in a qualitative manner and to optimize a quantitative assay for C-reactive protein (CRP).[206] Similar approaches to screen for biological analytes in a combinatorial fashion have also been developed using real-time biosensing detection schemes[200,207–217] or chip-based assays using beads.[218,219]

4. Autonomous (Microfluidic) Capillary Systems

Surface immunoassays comprise a number of steps, in which a surface is exposed to a series of solutions to provide reagents, analytes, rinsing, and these steps need extensive optimization in particular if the assays have to be sensitive and able to detect analytes from complex samples. In addition, drying in biology is a critical event. It is therefore desirable to have MFNs to miniaturize surface assays, as shown above, that can in addition flush microchannels or reaction chambers with multiple liquids without facing the risk of adventitious

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drying. There are many strategies based either on the design of the flow path itself, or using functional materials, or local actuation to program a few or all of these functionalities in microfluidics.[220–227] In general, actuated valves and flow regulators are used for controlling reaction times, mixing and dilution conditions, and metering samples. The concept of capillary systems (CSs) aims to address these challenges by encoding into the different sections of the CS (e.g., reaction chamber, access port, detection zone) the pumping and valving functions, which control the transport of liquid using capillary phenomena only (Fig. 4).[122] Here, aliquots for an assay can be successively added to the beginning of a CS in a zone termed the "loading pad". A chip can have many independent CSs in parallel, with each CS having its own capillary pump. Figure 4A shows an MFN having four such independent CSs. These CSs are three-dimensional (3D), and the chip can be placed anywhere on a PDMS surface without hindering access to the loading pads.

The capillary pressure along a flow path is set by changing its geometry, whereas its wettability can be kept equal everywhere. Aliquots are drawn from a loading pad to the end of the CS, which corresponds to the capillary pump by decreasing the capillary pressure along the flow path (Fig. 4B). Each functional element contributes to the overall hydraulic resistance of the CS, which affects the flow rate during the assay. The flow-rate resistance R_{FR} of a channel section for a CS shown in Figure 4A amounts to $R_F = 8.5 \times 10^{15}$ m^{-3}. The normalized R_F is shown in Figure 4B and reveals that here R_F accumulates mainly in the vias and the reaction chamber of the CS, and, importantly, that the contribution of the capillary pump is negligible because of its branching design. As the flow resistance is nearly constant in the capillary pump, this pump will draw aliquots successively added to the service port at a constant rate. The constrictions before and after the reaction chamber produce the lowest capillary pressure in the CS and act as a capillary retention valve to keep the reaction chamber wet at all times.

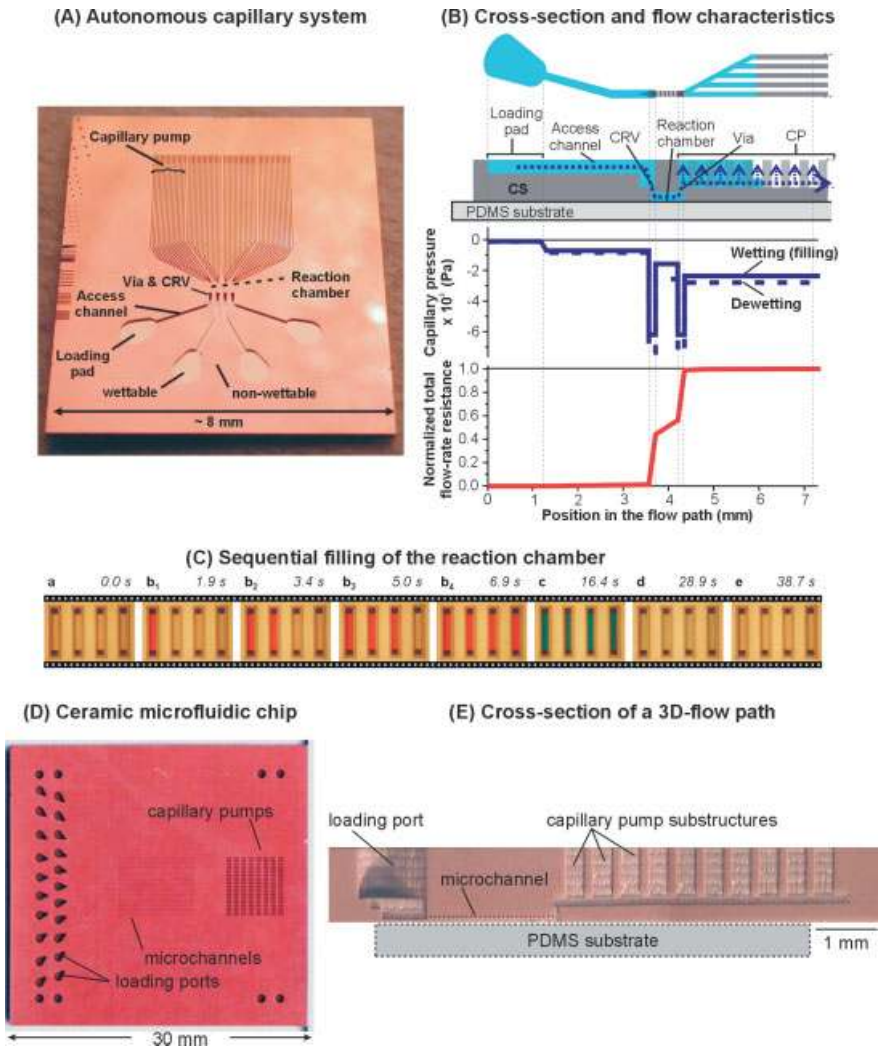

Figure 4. 3D autonomous CSs for the sequential delivery of liquids to a reaction chamber. A) Optical micrograph of an MFN, having four CSs, that was microfabricated in Si using multilevel photolithography and reactive-ion etching and that was then coated with a ~100 nm thick layer of Au. B) The capillary pressure along the flow path of a CS defines various functional elements. The region where the flow path is sealed by a PDMS layer corresponds to the reaction chamber, where the surface processing/assay takes place. The normalized flow resistance helps to predict the flow rate of a liquid filling the CS (CP: capillary pump). C) Images extracted from a video [228] showing 150 nL aliquots of water and water with dyes flowing sequentially through reaction chambers 500 μm long. D) Optical micrograph of a ceramic MFN having 21 CSs, where the loading ports, microchannels, and capillary pumps are coplanar and connected by embedded channels. Such chips are fabricated using multilayer ceramic technology. E) This cross section of a ceramic chip shows the 3D capillary path that brings liquids pipetted in the filling port to a microchannel on the opposite side of the chip and to a capillary pump. This chip was made using eight ceramic precursor sheets. ((A–C) were adapted with permission from [122]. Copyright 2002, American Chemical Society; (D, E) are courtesy of G. Natarajan and J. N. Humenik, IBM Server and Technology Group, East Fishkill, NY 12533, USA.)

Loading a series of aliquots into a CS is fast and convenient because the loading pads are a few millimeters square in area and wettable, although they are surrounded by hydrophobic areas.[229] It takes a few seconds to load multiple pads with sub-microliter volumes of solution by pipetting by hand. The flow in each CS is laminar, and aliquots can be flushed without excessive mixing (Fig. 4C) and with great efficiency because the CS is void of dead volume. The intrinsic capacity of the pump is ~800 nL, yet larger volumes can be flushed easily

by evaporating liquid in the pump using a stream of air[122] or heat as shown below. The flow rates with this design are on the order of 220 nL s^{-1}, which corresponds to flow velocities in the reaction chamber of ~55 mm s^{-1}.

There are a few techniques that are suitable for fabricating 3D microfluidic systems: multilayer lithography, mold replication (including soft lithography, hot embossing, and mold-injection techniques), multilayer ceramic packaging,[230] and solid-object printing.[125,231] Multilayer ceramic microfluidics are particularly attractive because they can comprise as many as ~110 layers, thus allowing complex CSs to be made, and they can have lateral dimensions of up to ~200 mm. Figure 4D shows a ceramic microfluidic chip having 21 3D CSs. This chip is a cordierite-based glass–ceramic, polished on both sides, wettable, and mechanically and chemically stable.[232] A slab of PDMS placed over the microchannels serves as a substrate for up to 21 independent assays. The cross section of the ceramic chip in Figure 4E illustrates well how multilayer ceramic chips allow complex flow paths to be formed. Three-dimensional microfluidic chips render sample-to-chip interfaces more accessible to users, pipetting robots, or peripheral equipment; they also provide the possibility to implement complex functional elements such as mixing units, heaters, filters, gates, actuators, and detection systems.[233–241]

4.1. Sequential Flushing for Miniaturized Surface Assays

The possibility of displacing series of liquids with laminar flow conditions on a precise region of a surface is very appealing, both for forming high-quality patterns of proteins on surfaces and for miniaturizing surface immunoassays. Figure 5 exemplifies these possibilities by showing highly accurate patterns of fluorescently labeled antibodies deposited on a PDMS surface as well as a surface fluorescence immunoassay for CRP. CRP is a 114 kDa pentameric protein synthesized by the liver. It is a widely used marker of inflammation and also an important prognostic indicator of coronary heart diseases.[242,243]

The making of high-quality binding sites on surfaces is crucial for many applications in life sciences and diagnostics, where biomolecules and analytes must be specifically identified and quantified in a variety of samples.[244,245] CSs are therefore well suited for applications that require a relatively small number of high-quality binding sites on a surface. This is the case for many diagnostic applications in which a few markers should be detected in parallel.[193,220] Reaction chambers, such as those shown in Figure 5A, can be cloned along one dimension at a density of ten independent chambers per millimeter. The well-known challenge of the world-to-chip interface[74,246] can be relaxed by fanning out the loading pads and capillary pumps along the periphery of the chip. CSs are flexible because any sequence of biological samples, buffers, and liquids for rinsing can be successively loaded to any loading pad of a CS. Such chips could even be used together with

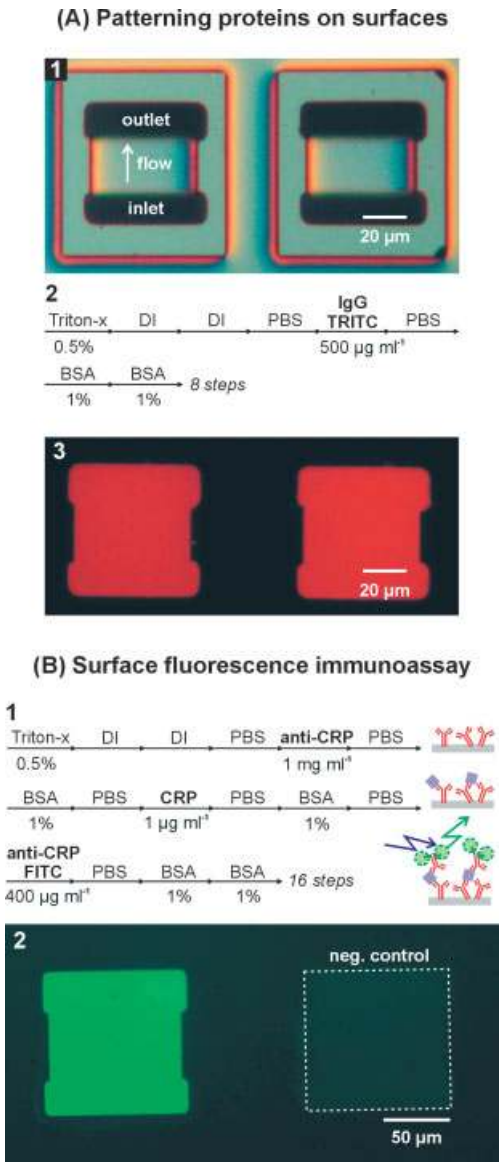

Figure 5. Patterning of proteins on a PDMS surface (A) and sandwich-type immunoassay (B) using CSs. A) The reaction chambers of two independent CSs, shown in (1) when viewed with a microscope through the PDMS substrate, were used to expose the PDMS surface to the series of aliquots listed in (2). Triton X-100; DI: deionized water. Some of the TRITC-labeled antibodies from the fifth aliquot adsorbed on the PDMS surface to form a highly accurate and homogeneous pattern as revealed in the fluorescence microscopy image in (3). B) A surface fluorescence assay to detect CRP was done using a large number of steps (1) and analyzed by measuring the fluorescence on the PDMS surface after separation from the microfluidic chip (2). The key to such a homogeneous patterning and assay are controlled flow conditions, the possibility to rinse the reaction chamber, and the absence of drying/evaporation effects before the deposition/assay is completed. (Adapted with permission from [122]. Copyright 2002, American Chemical Society.)

reagent-loaded cartridges.[247] Here, the incubation time for each step can be varied by adjusting the volume of liquid loaded into the pad or by changing the hydrodynamic properties of the capillary pumps. Real-time analysis of assays done

using CSs can be done by monitoring an optical signal through the PDMS surface[248] or by integrating a fluorescence-detection system into the PDMS.[249] The bioanalytical capabilities of CSs may be extended by including in the reaction chamber electrodes to perform assays using electrochemical detection principles,[250] cells to monitor cellular response to chemicals, or hydrogels for biosensing.[251–258]

4.2. Low-Volume and High-Sensitivity Assays

Assays in which analytes are present in a small volume of sample and at low concentration should greatly benefit from a microfluidic-based format because i) it is simple to fabricate a volume element of 1 pL (i.e., a cube with 10 μm sides) using conventional microfabrication techniques, ii) evaporation can be prevented or largely suppressed by diminishing air/liquid interfaces, iii) sensors and detectors (e.g., microelectrodes, charge-coupled devices, photomultiplier tubes, optical fibers, diffractive gratings) can be integrated in microfluidic chips or sufficiently miniaturized to probe surfaces $\ll 1$ mm^2 or volumes < 1 μL with high sensitivity limits, and iv) dead volumes can be prevented using appropriate designs and strategies to connect adjacent microfluidic elements.[73]

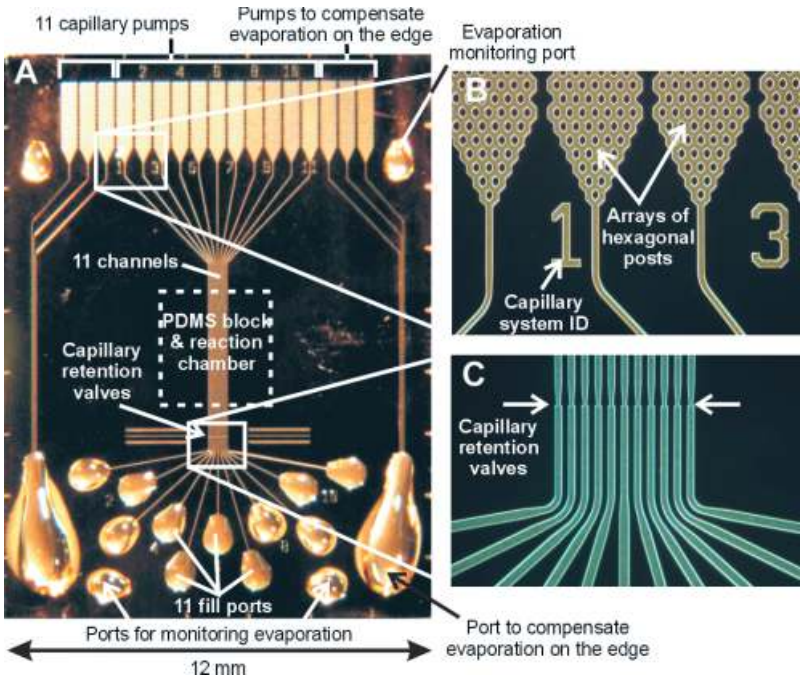

Figure 6. MFNs used for high-sensitivity, low-volume immunoassays. A) An optical micrograph of a ~2 cm × 2 cm MFN comprising eleven independent CSs, the filling ports of which are filled with ~300 nL of solution. A PDMS block is normally placed atop the microchannels to serve as the substrate for the assay. B) Arrays of hexagonal structures generate the capillary force in the capillary pumps. C) The capillary retention valves generate a strong capillary pressure at the beginning of the microchannels to retain liquid in the microchannel when the filling ports have been drained. (Reproduced with permission from [261]. Copyright 2004, Royal Chemical Society.)

Immunoassays find a practical limit in the ~1 pM range of analyte concentration owing to the limited affinity between antibodies and antigens.[193] Many analytes, such as cardiac markers, cancer markers, cytokines, and chemokines, require immunoassays having high sensitivities of 1 ng mL^{-1} down to 1 pg mL^{-1}. High-sensitivity assays also enable detection of new disease markers as well as understanding the biological role of low abundant proteins. Several applications require both high-sensitivity and low-volume assays. This is the case for some assays for in-vitro diagnostics, forensic applications, or for assays using cell cultures.[193,259] Access to key biological reagents unfortunately is sometimes a limiting factor for developing assays using biomolecules expressed by mammalian cell lines.[260]

Together with scientists from Hoffman-La Roche, we developed a high-sensitivity, low-volume assay for the tumor necrosis factor α (TNF-α) using MFNs.[261] The assay format is a micromosaic immunoassay done using two-dimensional CSs (Fig. 6). Chips were designed to have eleven or more open microchannels, each addressable by a filling port and ending with a capillary pump. The microchannels can be sealed by a PDMS slab. Loading pads are large enough to provide a convenient user interface, and a chip with 10–20 independent CSs has a footprint of 1–2 cm^2 and a thickness of 0.5 mm. Cooling the region in which the loading pads are located using a Peltier element minimizes the evaporation of aliquots during the time needed for the assay (10 to 20 min), and a heating element placed underneath the capillary pumps relays the pump once they are filled using evaporation. Flow rates as slow as 1 nL min^{-1} using forced evaporation can be achieved using this straightforward approach.[262] Evaporation also allows the pumping of volumes that exceed the capacity of the capillary pump.

TNF-α is a cytokine protein (molecular weight of 17 500 Da) that plays an important role in the generation and regulation of inflammation and immune responses.[263,264] Cytokines are measured in cell-culture supernatants for cytotoxicity studies,[261,265] in a variety of samples and organisms to understand cellular signaling,[266] and in the serum or plasma of patients for in-vitro diagnostics and for therapy monitoring.[193,267] TNF-α is, for example, found in healthy subjects at 1 pg mL^{-1} concentration, and higher concentrations may indicate health problems.[263] Figure 7A depicts the principle of the assay. A PDMS substrate is homogeneously coated with a capture antibody, blocked with BSA, and then placed over the set of microchannels. 600 nL of cell-culture supernatant is provided in each microchannel for a ~24 min capture step. The CSs are then flushed with rinsing solutions, and the PDMS is removed, dried, rotated, and placed across a second set of microchannels to deliver detection antibodies at various concentrations. After rinsing and drying, the mosaic of fluorescent signals is acquired using a fluorescence scanner.

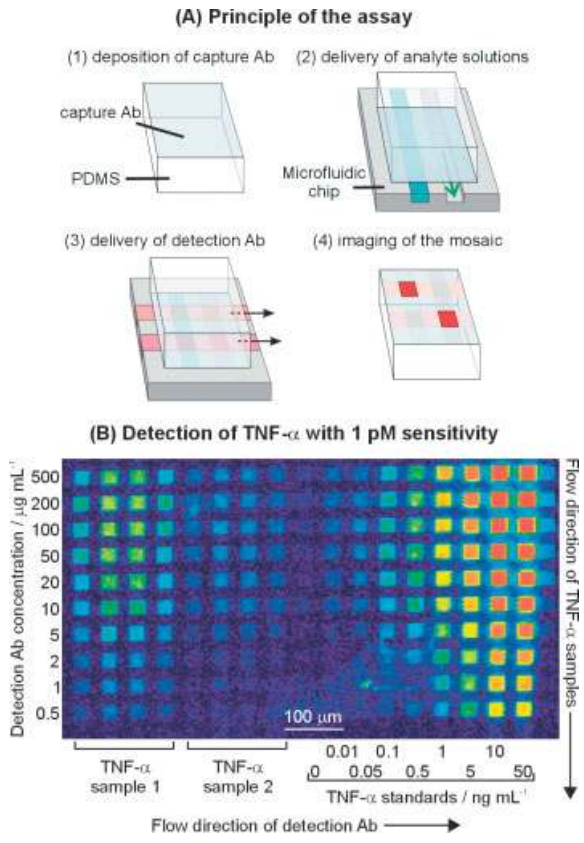

Figure 7. Micromosaic immunoassay for TNF-α. A) The assay uses two MFNs to localize the capture of TNF-α from solution to the PDMS surface and to provide detection antibodies at different concentrations. The first and second MFNs had 17 and 11 CSs, respectively, which resulted in a mosaic with a potential maximum of 170 fluorescent sites. B) This fluorescence scanner micrograph reveals the amount of TNF-α in the standard solutions (right) and in two supernatant solutions of T-cells that were activated with two factors (left). (Reproduced with permission from [261]. Copyright 2004, Royal Chemical Society.)

The sensitivity of this assay was found to be 1 pM (~20 pg mL^{-1}) and comparable with that of a state-of-the-art assay called dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA).[268,269] The micromosaic assay used only 600 nL of sample per line, and the entire mosaic was obtained in 45 min, whereas the DELFIA assay done using a microtiter plate necessitated 150 μL of cell-culture supernatant and 6–7 h. Generally, the detection limit of an assay is restricted by the background signal, i.e., the signal for zero analyte concentration.[193] Efficient blocking of the surface and rinsing are important to minimize the non-specific signal. Investigation of the fluorescence spectrum of PDMS revealed that it is best to employ dyes with long excitation wavelengths, and Alexa-647 was selected here for this reason.[261]

The minimum volume needed for a high-sensitivity low-volume assay is an important parameter to be considered.[2,270] In the previous example, 600 nL of TNF-α at a concentration of 1 pM contained only ~360 000 analyte molecules (0.6 attomoles). The smallest volume V_m needed to have at least one analyte in the sample is

$$V_m = \frac{1}{\eta N_a C} \tag{9}$$

where η is the signal detection efficiency and $0 \le \eta \le 1$, N_a is Avogadro's number, and C is the initial concentration of analyte in the sample. The binding reaction between a capture Ab and an analyte (antigen, Ag) in solution is characterized by the equilibrium $[Ab]+[Ag] \rightleftharpoons [Ab–Ag]$ and equilibrium constant $K = k_{on}/k_{off}$, where k_{on} ($s^{-1}\,M^{-1}$) and k_{off} (s^{-1}) are the association and dissociation constants, respectively. Typically, K ranges from 10^9 to 10^{11} for monoclonal Abs.[271] Although the binding of an antibody and antigen on a surface is complex and cannot be described using the same formalism as for binding reactions in solution,[193,194] it is possible to estimate the number of captured antigens as a function of time t, the surface density of "active" capture Ab Θ_{max}, K, and C using

$$\Theta_t = \frac{k_{on}\Theta_{max}C}{k_{on}C + k_{off}}\left(1 - e^{-(k_{on}C + k_{off})t}\right) \tag{10}$$

The maximum surface density of captured analytes at equilibrium $\hat{\Theta}_{bound}$ (for time $t \to \infty$) therefore is

$$\hat{\Theta}_{bound} = \frac{k_{on}\Theta_{max}C}{k_{on}C + k_{off}} \tag{11}$$

Note that Θ_{max} is affected by the average activity and orientation of the capture Abs on the surface.[272] From Equation 11, we see that at equilibrium and for initial analyte concentrations that are smaller than $1/K$, only a fraction of the analytes will be bound to capture Abs on the surface. Ultimately, the limit of detection is defined by the sensitivity of the signal-acquisition system, which for a biochip fluorescence scanner is of the order of a dye or less per μm^2. The binding of detection antibodies to surface-immobilized analytes usually is not a limiting step for sandwich surface immunoassays, because detection antibodies can be supplied in the microchannels at high concentration. Each capture site displayed in Figure 7 only needs ~100 captured analytes to generate a sufficient fluorescent signal for detection after the binding of the detection Abs. The benefits of miniaturizing the capture sites for fluorescence immunoassays are to i) potentially limit the depletion of analytes from solution,[273,274] ii) localize signals on well-defined areas, thereby facilitating their recording and processing,[121] iii) help the direct comparison of experiments in which one or several parameters of the assay are varied,[275] and iv) screen multiple analytes with minimal consumption of sample.[276]

From a practical point-of-view, ACSs are well suited for high-sensitivity assays because flow rates can be kept small to increase the duration of the capture step (reaction-limited step), several microchannels can be used for calibration and control purposes, and the reaction sites can be flushed with many rinsing solutions to minimize non-specific signal. A theoretical modeling of the assays shown here suggested that

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analytes at low concentration (≤1 nM) could be detected within seconds if all capture Abs were active and the flow rate of sample in the microchannel kept large enough to prevent depletion of analyte.[277] Strategies to pattern proteins inside microchannels[278] or to engineer binding sites using beads,[279,280] magnetic particles,[281–283] thermally responsive polymers,[284] oriented receptors,[285,286] conjugated proteins,[287] or self-assembly[288–290] would certainly augment the bioanalytical functionality of microfluidic systems.

Table 1 puts the characteristic dimensions of the main functional units of a CS into perspective with the characteristic timescale for diffusion in these units, and it enunciates the number of analyte molecules that can be accommodated in each unit.[2,184] The reaction chamber is the smallest-volume element of the CS, which helps increase the reaction rate for mass-transport-limited reactions. Diffusion, depletion, or excessive convection of analytes through the reaction chamber can each limit the performance of a miniaturized assay. Modeling an assay helps to delineate the most critical assay parameters, in particular when analytes are present in low concentration.[291–295]

4.3. Strengths and Limitations of Microfluidic Networks

As described above, MFNs can be reversibly sealed with a PDMS elastomer to localize the processing of the PDMS surface or miniaturize surface assays. The advantages of MFNs as compared with other microfluidics or surface-patterning methods reside in their powering by capillary forces; their macroscopic filling ports, which are easy to fill using conventional pipettes, empty automatically, and are free of dead volumes; the convergence of the reaction zones toward a small region of a surface for parallel assays; the possibility to program and vary flow conditions in the MFNs when they comprise arrays of CSs; the flexibility in loading these CSs with series of solutions; the high-resolution power of MFNs, and the simplicity of fabrication because the flow paths are only two-dimensional. The limitations of MFNs are probably similar to those usually encountered with microfluidic lab-on-a-CD and lab-on-a-chip platforms: numerous pipetting steps are needed when many assays are done in parallel; the large surface-to-volume ratios of microstructures can lead to depletion of reactants and analytes; diffusion-based mixing is not very efficient, and a gap exists between MFNs and conventional pipetting instruments in terms of the smallest volumes and positioning accuracies that can be achieved. In addition, the reversible sealing of MFNs with a substrate precludes processing non-elastomeric surfaces unless advanced strategies as described below are employed.

Table 1. Dimensions of various functional elements of a CS, related characteristic diffusion times, and number of moles of analyte molecules in the element.

	Entire chip	Loading pad	Capillary pump	Reaction chamber
Dimension [a]	1 cm	1 mm	100 μm	10 μm
Volume [b]	1 mL	1 μL	n × 1 nL	1 pL
Surface-to-volume ratio [c]	6×10^2 m^{-1}	6×10^3 m^{-1}	6×10^4 m^{-1}	6×10^5 m^{-1}
Diffusion time [d]				
Small molecule (urea)	20 h	12 min	7 s	72 ms
Small protein (TNF-α)	11.5 d	2 3/4 h	100 s	1 s
[analyte] [e]				
1 μM	10^{-9} mol	10^{-12} mol	10^{-15} mol	10^{-18} mol
1 nM	10^{-12} mol	10^{-15} mol	10^{-18} mol	10^{-21} mol
1 pM	10^{-15} mol	10^{-18} mol (TNF-α assay)	10^{-21} mol	"0.6" molecules

[a] Characteristic dimension of the functional element. [b] Characteristic volume defined as the cube of the characteristic dimension. [c] All six walls of the characteristic volume are considered, although one reactive wall only (processed surface or surface for assay) might play a role in depleting reactants from the volume. [d] Characteristic diffusion time for urea or TNF-α to diffuse across the characteristic volume using their respective diffusion coefficients at room temperature and in water of 1.38×10^{-9} m^2 s^{-1} and of 10^{-10} m^2 s^{-1}. [e] Moles of analyte molecules contained in the functional element for a given initial concentration.

5. "Open" Microfluidics

The term "microfluidic" stands as a summation of the extraordinary properties that liquids reveal when confined within microscale boundaries and of the use of these properties in miniaturized, microfluidic systems. "Microfluidics" has therefore become associated with the use of closed, miniaturized conduits. Yet, closed microsystems are difficult to interface with the macroscopic world, and microfluidic systems are therefore not suited for processing voluminous samples, large objects, and arbitrary surfaces. The remaining part of the review focuses on our approaches to confine and direct liquids efficiently on surfaces without using a closed conduit but having instead a geometrically open space.

One interesting concept to guide liquids between two proximal, parallel surfaces was realized by patterning wettable areas on a surface.[296,297] Electrowetting using an array of electrodes is another possibility for programming the wetting and displacement of a solution located between two plates.[298,299] Inkjet and other non-contact spotting technologies[300] are able to deliver nanoliter-sized drops onto surfaces, but they offer no control once the liquid has left the confinement of the ejection nozzle and, consequently, suffer from the problematic drying and spreading of the ink on the surface. Patterns of biochemicals can also be formed using pins or pen-like devices loaded with the appropriate solute and solvent to draw individual spots or lines.[301,302] However, the friction and resulting mechanical wear between the pen and the substrate during drawing are a concern and limit the resolution of writing techniques to a few hundreds of micrometers. The extension of the pen concept to the micro- and nanoscale using cantilevers derived from

atomic force microscopy can overcome this problem and is applied in dip-pen nanolithography[303,304] and related techniques.[305] Dip-pen nanolithography can pattern proteins and oligonucleotides with very high lateral resolution on a surface with minimal mechanical wear. The main drawbacks of pen-like devices for patterning surfaces are a lack of control after deposition of the material, the impossibility to rinse written patterns, and the difficulty to deposit different chemicals successively on the same area. Microfluidic probes, which we present in the next sections, combine many advantages of microfluidic systems with those of scanning probes and can be moved swiftly across a surface for lithographic purposes. We will first discuss how to confine a liquid on a surface and then present two implementations of the MFP concept that work in the presence of air or a surrounding liquid.

5.1. Confinement of Liquids on Surfaces Using Microfluidic Probes

Confinement of a solution on a surface without using lateral walls can be achieved by controlling the wettability of the surfaces in contact with the liquid.[296,297,306] Ideally, the confinement should not require modifying the substrate but should be independent of its wetting characteristics. A familiar device for patterning surfaces is the pen, which produces a thin line on a paper when it is continuously moved but forms an expanding spot when it is immobile. A slightly negative pressure generated by capillary structures holds the ink back in the pen in the absence of contact with a substrate. Spotters using pins rely on similar principles and also limit the contact time with the substrate. Figure 8 compares different confinement strategies for wettable and non-wettable substrates. Figure 8A represents a pen or pin-like device that has wettable surfaces. These devices typically have a tapered body and can be solid (AFM tip), incorporate a slit (pin with capillary microchannel), or comprise a bibulous material (pen). The spreading of the liquid when the device is static is limited if the substrate is hydrophobic or can extend well beyond the footprint of the device on the substrate if the substrate is hydrophilic.[307] When the device is displaced over the substrate, the wetting becomes dynamic, and the width of the capillary bridge depends on the velocity of displacement and withholding capillary pressure generated by device. In Figure 8B, the device has an opening with hydrophilic inner walls and hydrophobic external walls. This prevents the liquid from spreading when the substrate is non-wettable or hydrophobic.[123] In this case, small positive pressures can even be applied to the liquid without leading to uncontrolled spreading. The liquid can, however, spread along the gap when the substrate is hydrophilic. Figure 8C shows a general strategy for the efficient confinement of liquids in the absence of movement. This strategy consists of superposing appropriate wettability characteristics to the topography of the device such as to amplify the confinement power. Using this approach liquids can be confined on both hydrophobic and hydrophilic substrates.[308] The device comprises a mesa, having a hydrophilic front surface and hydrophobic lateral walls that pin the liquid at the periphery of the mesa. This configuration makes it energetically favorable for the liquid to wet the mesa and the underlying surface, which therefore occurs spontaneously, but energetically highly unfavorable for the liquid to wet the lateral

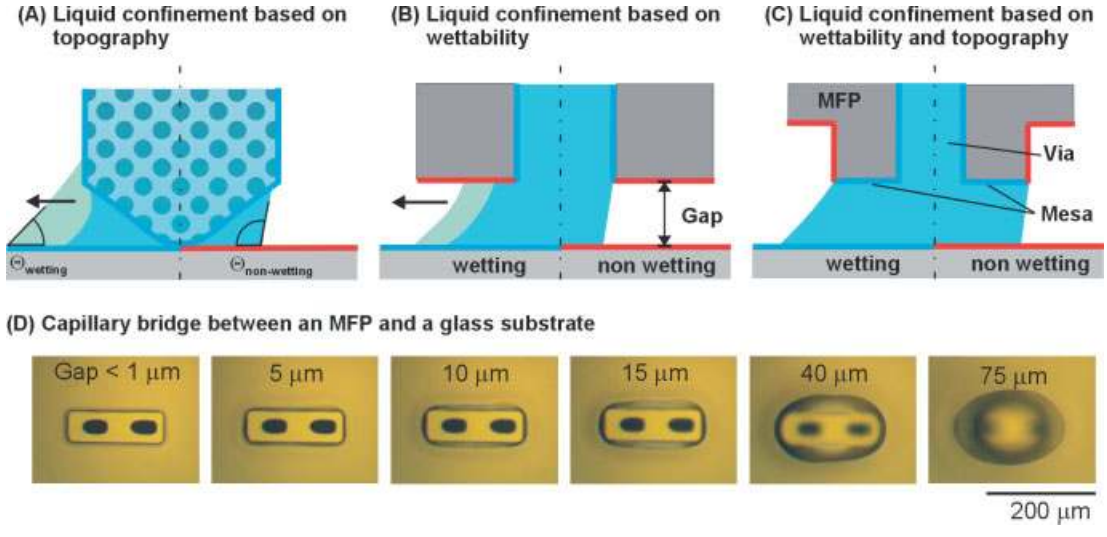

Figure 8. Confinement of a liquid between a patterning device and a substrate. Wettable and non-wettable surfaces are denoted by blue and red, respectively. A) Liquid confinement with pen-like devices mostly relies on the physical shape of the device and on movement to limit the spread of the liquid on hydrophilic surfaces. B) Liquid confinement can be based on the non-wettable properties of the patterning device and is successful for non-wettable surfaces. C) Adjusting the topography and wettability characteristics of a patterning device, such as MFPs, allows liquids to be accurately confined on substrates. D) These optical microscopy images show the confinement of water on hydrophilic glass surfaces using an MFP having two vias in the mesa as a function of the gap. The liquid matches the footprint of the mesa when the gap is ≤ 10 μm. The wetted substrate area increases gradually for larger separations until the capillary bridge between the MFP and the substrate breaks.

walls. The result is the automatic filling of the gap with liquid when the mesa is approached close to the surface and the pinning of the liquid at the edges of the mesa. Thus, a capillary bridge is formed between the mesa and the substrate surface. In this device, a via connects the mesa to a reservoir where a negative pressure can be created to further help confine the liquid in the gap. The volume within the capillary bridge and the wetted area on the surface depend on the height of the gap, the capillary pressure generated by the reservoir, the wettability of the substrate, and the geometry of the mesa, as can be seen in Figure 8D. Here, the substrate was a hydrophilic glass slide (advancing contact angle with water ~30°, receding contact angle 0°), and the liquid has a footprint on the substrate that closely matches the mesa for gaps up to 10 μm. This footprint increases gradually with the gap until the capillary bridge eventually breaks when the gap reaches ~75 μm, which is a dimension comparable to the microstructures present in the reservoir.

The wettability characteristics of MFPs are tailored by evaporating a thin, continuous layer of Au onto the devices. Self-assembling monolayers having either wettable (e.g., thiolated polyethylene glycol) or non-wettable end groups (e.g., hexadecanethiol) are patterned on the Au surface to form wettable and non-wettable areas, respectively.

An MFP can be operated as follows: first, a capillary bridge is spontaneously established by bringing the mesa into close proximity with a substrate. Subsequently, the gap is increased to the desired value, and the MFP is displaced parallel to the substrate. Further retraction of the MFP from the substrate leads to the breaking of the capillary bridge, which allows the writing process to be stopped. Writing patterns using fast lateral motion of the MFP can, however, lead to a rapid depletion of reagents in the capillary bridge, which can result in poor surface coverage. We review how this situation can be circumvented in the next sections.

5.2. Liquid Confinement and Laminar Flow: Microfluidic Probes Using Capillary Systems

Combining a CS and an MFP yields the possibility of creating a laminar flow of solution in a capillary bridge for processing substrates. In this case, the mesa must have at least two vias that serve as inlet and outlet, Figure 9A. The inlet is serviced by a loading pad, and the outlet is connected to a capillary pump. As in the MFP described above, a capillary bridge is formed when approaching the surface. However, this MFP is designed so that a flow of solution from the loading pad to the capillary pump occurs coincidentally with the formation of the capillary bridge; the flow is interrupted with the rupture of the capillary bridge by separation of the device. Here, the control of the flow is controlled by the approach and retraction of the MFP with the surface, whereas the flow in ACSs is triggered by loading a solution into a loading pad. Both the loading pad and the capillary pump of the MFP are structured; the structures in the capillary pump are smaller and thus generate a stronger pressure, which establishes a unidirectional flow from the loading pad towards the pump. The flow rate is de-

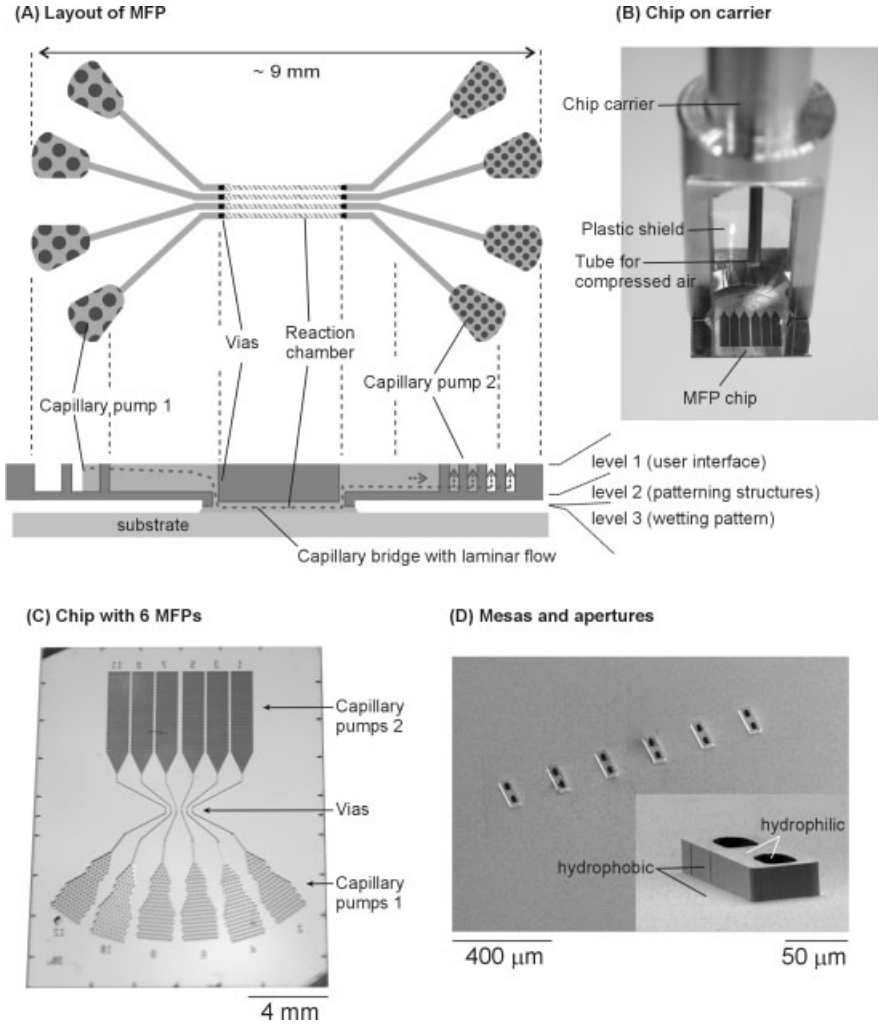

Figure 9. MFPs incorporating CSs are able to process a substrate with a laminar flow of solution. A) The capillary bridge formed between the mesa of an MFP and the substrate is supplied with solution from a loading pad, which flows to a capillary pump. B) Microfabricated chips comprising multiple MFPs can be mounted on a carrier for convenient handling and patterning. C) Photograph of chip having six independent MFPs. D) Scanning electron microscopy images of the bottom side of the chip in (C), showing the mesas.

fined according to Equation 1. Interestingly, the flow-rate resistance can be finely tuned during operation by varying the gap between MFP and surface.

Several MFPs can be cloned on a chip. Figure 9B shows a chip having six MFPs affixed to a carrier, which can be mounted to a computer-controlled *X-Y-Z* stage. The image in Figure 9C shows an MFP ~2.4 cm × 2.4 cm in size with six independent loading pads and capillary pumps. The six corresponding mesas are shown in Figure 9D. The mesas are 60 μm wide, 180 μm long, and spaced 200 μm apart.

Figure 10 illustrates the patterning of a glass substrate using a 0.5 mg mL^{-1} solution of TRITC-labeled Abs in PBS. In addition, the solution contained 2.5 μm large fluorescent beads that were used as flow tracers. The glass substrate was hydrophilic (clean and untreated) to demonstrate that the capillary bridge is indeed stable, although a hydrophobic substrate would have promoted better adsorption of the proteins from

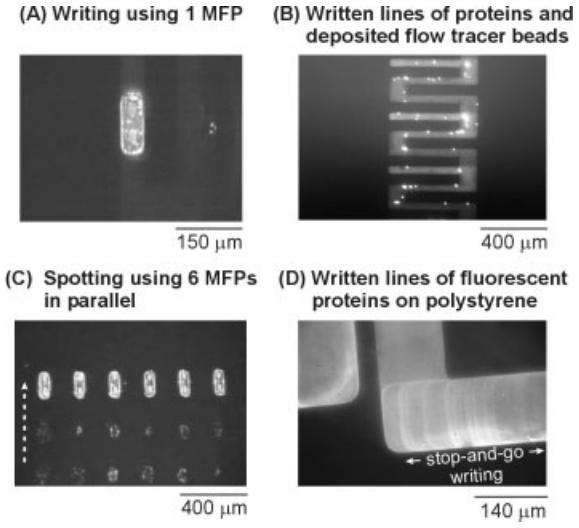

Figure 10. Fluorescence microscopy images that show patterns of fluorescently labeled proteins deposited from solutions flowing in a capillary bridge formed between an MFP and a surface. A) Several images from a video sequence were overlaid to visualize fluorescent beads flowing in the capillary bridge of an MFP. The beads reveal the mesa of an MFP and a U-shaped line of TRITC-labeled Abs deposited onto glass. B) TRITC-labeled Abs were patterned on a hydrophilic glass surface by displacing an MFP with varying velocity. The bright objects are fluorescent beads, which were used as flow tracer and which occasionally attached to the glass. C) An array of six MFPs is used to write spots of proteins on a glass slide. D) FITC-labeled Abs were patterned on a hydrophobic polystyrene substrate with high edge resolution using an MFP.

solution. Figure 10A represents a series of fluorescence microscopy images that were overlaid for visualization purposes: the mesa of the MFP appears bright owing to the presence of beads moving in the capillary bridge. The pattern of protein seen here has a U-shape, which exhibits sharp borders and reveals the patterning resolution of this technique. The image in Figure 10B shows a series of U-shaped patterns, over which some tracer beads were deposited. MFPs can be utilized in parallel, as shown in Figure 10C, in which the MFPs were brought close to the substrate to form the capillary bridges, left on the sites for ~1 s, and moved to subsequent sites in ~0.1 s. Figure 10D shows a high-resolution fluorescence image obtained by depositing FITC-labeled Abs from a 1 mg mL^{-1} solution onto a hydrophobic polystyrene substrate. In this experiment, the microscope table supporting the substrate surface was manually displaced underneath an MFP. Remarkably, the resulting lines exhibit an edge resolution of better than 5 μm. The fluorescent stripes inside the lines originate from stop-and-go movements that were applied to the chip during this writing operation and from convection-driven deposition of proteins at the edge of the capillary bridge. Typical writing speeds were ~100 μm s^{-1}, and speeds up to 1 mm s^{-1} can be achieved before the capillary bridges break.

The key features of MFPs working with a capillary bridge in the gap that separates the microfluidic device from a substrate are that i) MFPs can be used to process a large surface with a solution by scanning over the surface, ii) they operate in a non-contact mode and need no sealing, iii) the capillary bridge forming under the mesa has a controlled geometry, iv) the flow below the mesa is laminar, and transport in the thin capillary bridge is very effective, v) the flow of the processing solution is triggered automatically when the MFP approaches the substrate and stops after it retracts, and vi) they are therefore very economical of reagents. MFP technology could be valuable for arraying biomolecules as well as for general patterning applications, for example, for writing a seed layer in the shape of lines for subsequent metallization.[309,310]

6. Non-Contact Microfluidics Operating Under a Liquid

The MFP concept can be extended to work in a liquid, which can be advantageous for processing biological specimens in solution under physiological conditions, for working with surfaces that are sensitive to air, or for carrying out reactions at a liquid–liquid interphase.[91,92,311] The presence of a liquid in the gap between the MFP and a substrate can also prevent problems with evaporation that are recurrent in the fabrication of biochips. Nanopipettes have recently been used to write high-resolution DNA and protein patterns on surfaces in the presence of a surrounding liquid and with the use of electrical fields to control the flow of reagents from the pipette tip to the surfaces.[312,313] Coaxial pipettes were also used for releasing neurotransmitters to a neuronal network with spatial control.[314] These two techniques are elegant but may lack versatility and convenience for a range of applications.

The next two sections show how to confine a processing solution in a laminar stream using hydrodynamic forces and illustrate the possibilities of this concept with various additive and subtractive surface-patterning examples.

ADVANCED MATERIALS

6.1. Microfluidic Probes for the Hydrodynamic Confinement of Liquids

Figures 11A,B show the flow profile of a solution that is being injected from one aperture of the mesa into a gap containing a surrounding liquid, and aspirated together with some of the surrounding liquid by a second aperture of the mesa. If the injection flow rate (Q_I) is significantly lower than the aspiration flow rate (Q_A), the injected solution is deflected, confined and focused by the concentric flow field into a microjet, and then directed into the aspiration aperture, as shown here. We define the confinement of a processing solution by a surrounding liquid in a geometrically open space as hydrodynamic flow confinement (HFC). HFC bears similarities to hydrodynamic focusing,[7] because a first fluid is being enclosed and focused by a second one, but is different in that there are no physical sidewalls. Therefore, if the ratio Q_A/Q_I falls below a critical value, defined as the confinement limit, the confinement is lost, and the injected fluid leaks into the surrounding medium. The hydrodynamic focusing strength can be adjusted by the ratio Q_A/Q_I, and, importantly, for small gaps, the microjet can be forced to impinge on the surface. The variation of Q_A and Q_I, in conjunction with the gap size, can be used to fine-tune the shape and size of the impingement area on the surface, thus, enabling a processing solution to flush the surface and either deposit or remove material locally. The fluorescence microscopy image in Figure 11C shows a liquid colored with fluorescein and containing red fluorescent beads flowing from one aperture to another during 8 s. The flow velocity is slower at the periphery of the flow field, which translates into red lines of increased intensity at the periphery.

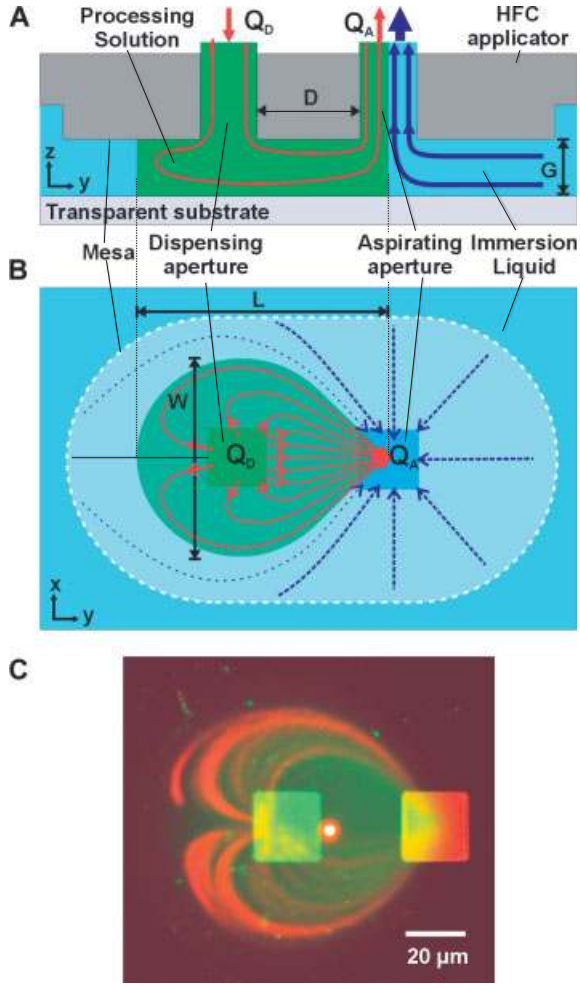

Figure 11. MFP used for the HFC of a liquid over a surface. A) Cross-sectional and B) bottom views of an MFP in an immersion liquid and over a substrate. The immersion liquid is aspirated into an aperture at a flow rate Q_A (blue area, blue flow lines) and exerts a hydrodynamic pressure on a stream injected through a second aperture at a rate Q_I ($< Q_A$). The stream is deflected, confined and focused into a microjet (green area, red flow lines), and guided into the aspiration aperture. C) Fluorescence microscopy image, recorded during 8 s, that shows the flow path of a liquid between the apertures of an MFP and over a glass substrate. The aqueous microjet contained fluorescein (green) and 2.5 µm wide fluorescent beads (red) that reveal the shape and flow lines, respectively, whereas the immersion solution (water) appears black. Here, the gap was 10 µm, $Q_I = 0.44$ nL s^{-1}, and the ratio $Q_A/Q_I = 2.5$ resulted in a relatively wide microjet. (Reproduced with permission from [124].)

6.2. Processing Surfaces with Microjets Using a Microfluidic Probe

HFC combined with the capability to scan the MFP across a surface permits the writing of arbitrary patterns on surfaces using a continuous delivery of reagents. When the substrate is scanned relative to the MFP, a laminar drag, a so-called Couette flow, develops between the mesa of the MFP and the surface and is superposed to the concentric flow field. Controlled use of this effect allows the processing of isolated areas of a surface with a simple stop-and-go movement without having to interrupt the flow of the processing solution or retract the MFP from the surface. Indeed, when the substrate is moved in the direction of the microjet flow, the microjet is being viscously dragged towards the aspiration aperture, and a surface boundary layer of immersion liquid "isolates" the surface from the microjet during the brief passage under the mesa. A high-density array of antibodies was formed on a glass by taking advantage of these hydrodynamic phenomena (Fig. 12A). When the substrate is moved against the direction of the microjet, the Couette flow opposes the flow direction of the microjet. Moderate velocities slightly enlarge the confinement envelope of the jet, and molecules will be deposited as a line, which occurs here on the first two spots of each vertical line. The two types of antibody of the array were patterned in less than 15 min each, using ~300 nL of solution, and have a surface density of $>15\,000$ spots cm^{-2}. A 2–5 µm gap between MFP and surface, to ensure high mass transport and rapid adsorption of the proteins to the surface, resulted in a spot being formed in 0.3 s by using only 130 pL of protein solution. Compared with conventional spotting,[315] sample consumption here is as low as in those techniques, whereas spot uniformity and density are superior.

A continuous variation of the scanning velocity of the MFP can be used to modulate the density of adsorbed proteins and to form continuous microscale gradients (Fig. 12B), which can be localized arbitrarily on the surface. Reactions that are sufficiently fast compared with the diffusion rate of reactants can be carried out at the interface between the injected and the surrounding liquids. In Figure 12C, such a reaction was produced using HFC. Here, fluorescently labeled proteins that were dissolved in the microjet precipitated at the interface to the immersion solution (deionized water). Varying the ratio Q_A/Q_I, the gap between the MFP and the surface, as well as displacing the MFP all influence the resulting pattern of precipitated protein. In contrast to most conventional wet-patterning techniques, MFPs also allow subtractive processes, as shown in Figure 12D, where proteins were desorbed from a surface. Here, the injected processing was formulated for the rapid detachment and dissolution of the proteins from the surface of a glass slide. A microjet can be confined over a cell adhering to a surface for staining or removal purposes. The microscopy images in Figure 12E show the selective removal of a single cell from a surface, without affecting adjacent cells, by locally exposing the cell to a solution of trypsin. Addressing single cells on a surface may find applications in, for example, diagnostics, the study of intra- and intercellular signaling pathways, the analysis of tissue sections, and cell–drug interactions.[316]

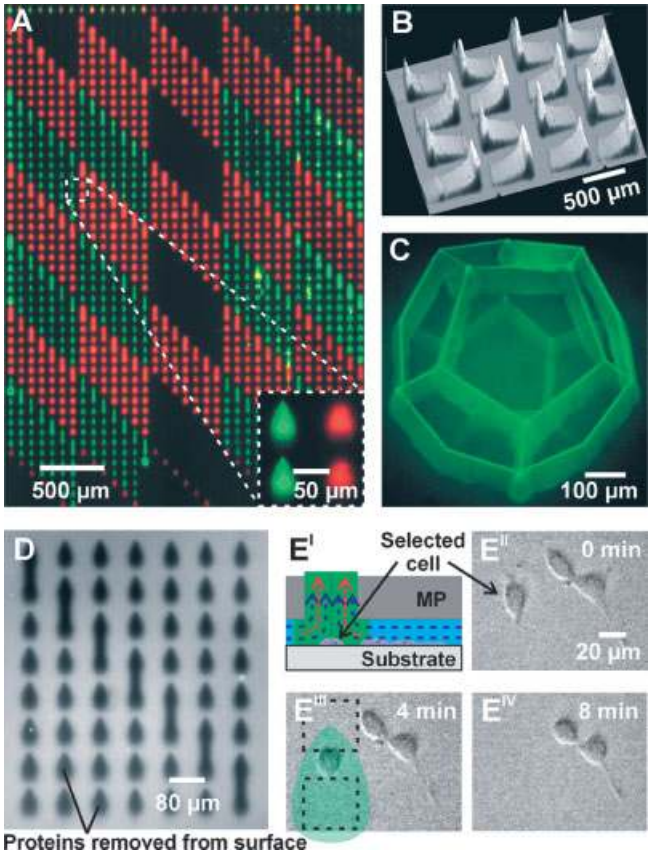

Figure 12. Additive and subtractive patterning of surfaces using an MFP in solution. A) Fluorescence microscopy image of a protein array featuring 1384 spots spaced 80 μm apart. Two types of protein were subsequently patterned. The first protein was a TRITC-labeled Ab (red), and the second was an Ab (from rabbit) stained with a FITC-labeled anti-rabbit Ab (green). The inset reveals the high quality of the sites. B) Fluorescence intensity profile of a 4 × 4 array of fluorescently labeled proteins deposited with a surface-density gradient on a glass surface. The gradient of proteins on the surface reflects the velocity gradient of the MFP. C) Fluorescence microscopy image of a dodecahedron written using FITC-labeled Abs that precipitated at the interface between the laminar microjet and the immersion medium (deionized water). D) Fluorescence microscopy image of a layer of TRITC-labeled Abs on glass, for which an MFP was used to remove some of the adsorbed Abs selectively from the surface. E) A single, adherent fibroblast cell in a cell culture was selected and detached from the substrate by flushing a jet containing trypsin over the cell. (Reproduced with permission from [124].)

6.3. Opportunities and Challenges for Microfluidic Probes

Processing surfaces using microfluidics in non-contact mode opens up a range of new opportunities because a solid, closed microchannel is no longer needed. Many more applications can therefore benefit from the advantages and specific characteristics of microfluidic systems: fast reactions/processing rates can be achieved, only small volumes of reagents are needed to process large areas, and the contact area of the surface-processing liquid can be accurately controlled to below a few μm^2. In addition, both additive and subtractive processes are possible. The non-contact microfluidic technology is versatile, but its implementation is still at an early stage, and further refinement is needed to turn it into a robust and practical tool. The fabrication remains difficult, as the confinement of the liquid in the presence of an air gap requires MFPs to have both a wetting and a topographical pattern. MFPs are three-dimensional and therefore necessitate forming through-wafer vias and multilayer lithography. In the presence of a liquid, the MFP must be interfaced to active pumps so that the flow of the dispensed and aspirated solutions can be adjusted as needed. Controlling the separation distance between an MFP and a surface during scanning remains a difficult issue for non-planar surfaces and is challenging for so-called planar surfaces when square-centimeter-scale areas are to be treated having micrometer-sized gaps between the MFP and the surface. However, by borrowing technologies developed in the field of scanning probes, dynamic and reliable control of the position of the MFP above the substrate seems feasible.

7. Concluding Remarks

As is true for many new technologies, bold predictions have been made in the 1990s about "shrinking" chemistry and biology, and conceiving biomedical microdevices using microfluidic chips. The complexity of real life has caught up with the initial enthusiasm, and neither in research laboratories nor in doctors' offices has microfluidics been successful at replacing even a few of the conventional technologies within such a

short timeframe. This is in stark contrast to microarray spotting, which may be considered as the most elementary form of microfluidics, and which has become a widespread technique in life-sciences research largely because of its ease of parallelization. Whereas by using a pin spotter it has become possible to readily transfer volumes of the order of a few picoliters, the precise delivery of a few nanoliters of solutions into microfluidic systems remains a challenge. Large dead volume, sensitivity to clogging, poor dispensing accuracy and reproducibility because of uncontrolled capillary effects are some of the plagues that limit today's dispensers. In addition, scalable solutions for parallelized dispensing are still missing. One important cause for such a situation is that microfluidic systems are themselves prone to these problems.

Microfluidic systems based on capillary effects described in this review can help address some of the present issues in microfluidics, because of their straightforward interfacing with a variety of liquid-handling devices and with conventional data-acquisition systems, their self-powered properties, and their general ease of use. Their scalability may further provide an incentive for creating parallelized and robust microfluidic dispensing systems. The coding of fluidic functions with capillary pressure is the key and certainly bears the potential for further refinement by integrating more functionality so as to automatically perform complex operations with solutions. Capillary systems, with their simplicity, offer many advantages: they can, for example, also be used for chromatographic separations,[317] and they seem particularly promising for applications in research in life sciences.

The MFP concept broadens the notion of microfluidics beyond the microchannel by superseding microconduits and the necessity of introducing the sample into such conduits. The use of MFPs can enhance existing applications, such as patterning and processing surfaces, which benefit from microfluidic conditions but which were traditionally limited by the necessity of using closed microchannels. The MFP may also cover some applications traditionally reserved to microarray spotters as it has the advantage of controlled conditions, but with the challenge of parallelization remaining. Single-cell manipulation serves as an illustration of the versatility of the MFP concept and may certainly be extended to the processing of a large variety of surfaces and biological specimens.

Chemistry, biology, and even medicine all converge on the trend of miniaturization, which creates a demand for microfluidic solutions. It therefore seems certain that microfluidics will impose themselves in an increasing number of routine applications in these disciplines. The field of microfluidics itself is deeply rooted in a multidisciplinary research environment, and microfluidics have already started extending into yet other fields, such as materials science and bioengineering, and one may therefore expect novel functions, applications, and opportunities to arise in the near future.

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