Patterning Multiplex Protein Microarrays in a Single Microfluidic Channel

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Supporting Information

ABSTRACT: The development of versatile biofunctional surfaces is a fundamental prerequisite in designing Lab on a Chip (LOC) devices for applications in biosensing interfaces and microbioreactors. The current paper presents a rapid combinatorial approach to create multiplex protein patterns in a single microfluidic channel. This approach consists of coupling microcontact printing with microfluidic patterning, where microcontact printing is employed for silanization using (3-Aminopropyl) triethoxysilane (APTES), followed by microfluidic patterning of multiple antibodies. As a result, the biomolecules of choice could be covalently attached to the microchannel surface, thus creating a durable and highly resistant functional interface. Moreover, the experimental procedure was designed to create a microfluidic platform that maintains functionality at high flow rates. The functionalized surfaces were characterized using X-ray photoelectron spectroscopy (XPS) and monitored with fluorescence microscopy at each step of functionalization. To illustrate the possibility of patterning multiple biomolecules along the cross section of a single microfluidic channel, microarrays of five different primary antibodies were patterned onto a single channel and their functionality was evaluated accordingly through a multiplex immunoassay using secondary antibodies specific to each patterned primary antibody. The resulting patterns remained stable at shear stresses of up to 50 dyn/cm². The overall findings suggest that the developed multiplex functional interface on a single channel can successfully lead to highly resistant multiplex functional surfaces for high throughput biological assays.

Precise microfluidic control and manipulation of liquids in geometrically controlled miniaturized channels has proven a useful tool for developing Lab-on-a-Chip (LOC) devices. Among the microfluidic platforms under development, multiplex detection interfaces, created by patterns of different biomarkers or multiple stimuli in a single LOC device, has attracted much attention for high throughput biological applications such as biosensing and cell detection. To develop biofunctional LOC devices, spatial positioning and orientation, as well as binding of functional biomolecules to substrates should be well controlled. Localized and region specific patterning of multiple biomarker microarrays within microfluidic platforms is another important feature of a functional interface for the production of multiplex high-throughput devices.

Despite promising progress in developing functional surfaces in LOC devices, many limitations remain in terms of producing highly resistant devices with durable multiplex detection interfaces using cost-effective and straightforward techniques. Several approaches have been introduced to pattern biomarkers on microchannel surfaces. Among them, microfluidic patterning and microcontact printing are widely used since they are simple and rapid to implement. Microfluidic patterning involves using laminar flow streams to functionalize surfaces, though results are limited to geometrical patterns along the streamlines. This limitation can be overcome by soft lithographic approaches, such as microcontact printing using different stamp designs for region specific functionalization. However, common techniques used to irreversibly bind the microfluidic substrates (e.g., plasma treatment) are destructive to the microcontact printed patterns. To protect the contact-printed surfaces, a poly(dimethylsiloxane) (PDMS) stamp can be placed on the surface during plasma treatment or an opaque box of PDMS can be used. These techniques are either time-consuming or require additional steps to arrive at an irreversibly sealed microfluidic device.

The robustness of the functionalized interface is another important consideration. Many platforms developed via microcontact printing rely on physical adsorption of biomolecules to the substrate. Protein patterns formed in this manner tend to detach from surfaces and are in turn particularly problematic in microfluidic systems operating under various flow conditions. The efficacy and durability of such patterns may also be altered for long-term applications. Covalent binding using silane solutions, which create active groups on microfluidic substrates for the subsequent attachment of biomolecules, is usually employed to address this limitation. The requirement for air free environments, and the need to work under nitrogen or argon chambers, reduces

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the versatility of the silanization technique for the development of time effective interfaces.

For multiplex patterning, robotic printing is the most commonly used technique with which different ligands can be patterned onto a small area. The advantage of this technique resides in patterning different biomarkers onto a small area, though the system is very costly and occupies a large space.

In this work, a dual approach using a combination of microcontact printing and microfluidic patterning is implemented to create highly resistant multiplex detection interfaces in a microfluidic platform. A protocol for microcontact printing of (3-Aminopropyl) triethoxysilane (APTES) on glass substrates is introduced as a promising approach for rapid and simple printing of functional groups to create region-specific patterns. Simulation of the flow conditions and diffusion between laminar flow streams containing different concentrations of biomolecules was performed to obtain experimental conditions in which multiplex probes are produced within a single channel. The proposed approach provides the following advantages: (i) creates a multiplex detection interface on a single channel for high throughput biological applications; (ii) uses silane microcontact printing to introduce amine functional groups onto the microchannel surface as a means to covalently immobilize the biomolecule of choice, thus overcoming the problems posed by the physical immobilization of biomolecules such as resistance to shear stress; (iii) drastically reduces the time and effort required for silane patterning of the surface; (iv) results in a microfluidic device capable of withstanding high flow rates through irreversible binding; (v) controls the patterning outcome by flow conditions and concentration of the proteins in each flow, in which the patterned probes could possess single or dual antibodies.

2. MATERIALS AND METHODS

2.1. Reagents and Materials. The negative photoresist SU8-2025 was purchased from Microchem Corp (Boston, MA). (3-Aminopropyl) triethoxysilane (APTES), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), phosphate-buffered saline (PBS), hydrogen peroxide, bovine serum albumin (BSA) and sulfuric acid were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Sylgard 184 elastomer kit composed of prepolymer and curing agent of poly(dimethylsiloxane) (PDMS) was purchased from Essex Chemical (Boston, MA). Mouse Anti-CD34, rabbit Anti-CD-31 and rat Anti-CD36 primary antibodies, anti-rabbit Cy3 conjugated IgG secondary antibody, anti-rat Cy5 conjugated IgG secondary antibody, and anti-mouse fluorescein isothiocyanate (FITC) conjugated IgG secondary antibody were purchased from Abcam (Cambridge, MA).

2.2. Design and Fabrication of the Microfluidic Device. Microfluidic design for channel systems (μFN) and stamps for microcontact printing were generated using AutoCAD software (Autodesk Inc., San Rafael, CA). The device consists of seven input microchannels, 100 μm in width and 60 μm in depth merging into a main channel in three different areas (Figures 1E and H). The design was printed on a chrome mask. To fabricate the mold for soft lithography, negative photoresist (SU-8 2025) was spin-coated (at 1500 rpm for 30 s) on a silicon wafer and baked to drive off solvent. Photolithography was performed on the wafer using the printed
mask to fabricate the mold. The mold was used to create the PDMS platforms. The protocol for creating the irreversibly sealed microfluidic platform is described in section 2.6. Once the closed channels were formed, the inlet and outlet tubes were connected. Flow control was achieved using a multi-stage syringe pump (Nexus 3000, Chemyx Inc. Stafford, TX). 18G syringe needles (BD, ON, Canada) were cut and used as inlet and outlet connections. Modified pipet tips were used as a support to fix the inlets and outlets by epoxy glue (Figure 1H).

2.3. Simulation. Simulation of concentration distribution in the device was performed using COMSOL (COMSOL, Inc. Burlington, MA). The Computational Fluid Dynamics (CFD) analysis was first performed to solve the continuity (eq 1) and Navier–Stokes (eq 2) equations:

\[
\nabla \cdot \mathbf{u} = 0
\]

\[
\frac{\rho \partial u}{\partial t} - \nabla [\eta \left( \nabla u + (\nabla u)^T \right)] + \rho(u, \nabla)u + \nabla p = F
\]

in which \( \eta \) is the dynamic viscosity \( \rho \) density, \( u \) is velocity vector, \( p \) is pressure, and \( F \) is volume force field. Equation 2 can be simplified by assuming steady state conditions and volume forces equal to zero. Species diffusion in the solution was then modeled using multiphysics properties of the software. The velocity components from the CFD results were simultaneously implemented for concentration distribution using the convection and diffusion module. Species diffusion in flow conditions was solved according to eq 3:

\[
\frac{\partial c}{\partial t} + \nabla \cdot (-D\nabla c + cu) = 0
\]

in which \( c \) represents each species concentration and \( D \) is the diffusion coefficient (about \( 1 \times 10^{-11} \text{ m}^2 \text{ s}^{-1} \) for proteins). The geometrical model was generated in AutoCAD software and was imported into COMSOL interface. The fluid inside the channels was assumed to have the properties of water (Newtonian fluid) and a no-slip boundary condition was applied. Inlets and outlets were specified as the entire opening at the beginning and end of each geometry, as opposed to the actual microfluidic device in which inlet holes are in the channel roof. This assumption only changes the flow profile at the inlets and outlets of each channel and has no effect on the velocity/concentration profile within the channels.31

2.4. Microcontact Printing of APTES on Glass. Different types of PDMS stamps were used for APTES microcontact printing: flat stamps to create uniform amine groups on the surface (section 2.6.1) and stamps with patterns to create independent biofunctional spots (sections 2.5 and 2.6.2). The PDMS stamp was plasma treated for 1 min using oxygen plasma (60 s, 200 W, 200 mTorr \( \text{O}_2 \)). It was then covered with 10 \( \mu \text{L} \) of 2% APTES solution in ethanol at room temperature. To avoid evaporation of the APTES solution, a coverslip was placed on the stamp for 1 min. After rinsing with 70% ethanol solution for 15 s and drying under nitrogen gas for 30 s, the stamp was gently brought into contact with the predetermined areas on glass substrate for 5 s. Immediately after printing the APTES solution, the plasma treated PDMS substrate (μFN) was brought into contact with the microcontact printed glass substrate to form the microfluidic device as described in section 2.6. The device was then placed in an oven at 100 °C for 60 min to ensure covalent binding of APTES with glass proceeded through condensation of hydrogen bonded silanol groups (Figure S1, Supporting Information).

2.5. Region Specific Surface Patterning in Stop Flow Conditions. Microcontact printing and glass surface functionalization were initially performed in stop-flow conditions to optimize the surface functionalization procedure. APTES was contact printed according to the protocol described in section 2.4 using the desired patterns fabricated on PDMS stamps. The substrate was then cured in an oven at 100 °C for 1 h. NHS (5 mg/mL) was used along with EDC (2 mg/mL) to cross-link the biomolecules to the amino-silanized patterned surface (Figure S1, Supporting Information). A 200 \( \mu \text{L} \) aliquot of FITC conjugated IgG and Cy3 conjugated IgG secondary antibodies were placed in contact with the surfaces for 60 min at a concentration of 10 \( \mu \text{g/mL} \). After rinsing the surface with PBS, the patterned surfaces were analyzed using a fluorescence microscope (Nikon TE 2000-E).

2.6. Surface Functionalization Procedure Using Microfluidics. Glass substrates were placed in piranha (\( \text{H}_2\text{O}_2/\text{H}_2\text{SO}_4, 1:3 \text{ v/v} \)) solution for 10 min, rinsed extensively with DI water, and dried under a stream of nitrogen. As shown in Figure 1, two substrates forming the microfluidic device were plasma treated (60 s, 200 W, 200 mTorr \( \text{O}_2 \)) followed by microcontact printing of APTES onto the glass substrate. Immediately after microcontact printing, the glass and patterned PDMS substrates were attached to form an irreversibly sealed microfluidic device. After heat treatment at 100 °C for 1 h, EDC-NHS solution was flowed into the printed area and incubated over the main channel for 20 min to activate the printed amine groups on the glass side of the microfluidic channel. Separate flow streams containing different antibody solutions were then passed through the input channels (Figure 1) and merged into a main channel. The antibodies in each flow stream were covalently bound to the printed amine groups on the surface (Figures 1D and 1F). Surface functionalization with multiple antibodies using the procedure described in section 2.6.1 was performed, and functionality of the patterned antibodies was investigated (section 2.6.2).

2.6.1. Multiplex Protein Functionalization. After microcontact printing of APTES onto the glass substrate and immediately binding it to the PDMS substrate, an irreversibly sealed microfluidic platform was formed. The PDMS stamp used for this stage is a flat stamp with no pattern and wide enough to cover the main channel area. After connecting the inlets and outlets, the device was washed with a flow of PBS and the printed APTES molecules were activated by EDC-NHS. Laminar flow streams of antibodies, namely Cy3 conjugated IgG to rabbit, Cy5 conjugated IgG to rat, and FITC conjugated IgG to mouse secondary antibodies, were then flowed for one hour through separate input channels and directed to the main channel at a flow rate of 0.5 mm/s. The channel was then rinsed with PBS at a flow rate of 10 cm/s (50 dyn/cm²).

2.6.2. Multiplex Protein Microarrays in a Single Channel and Immunoassay. To create multiplex protein microarrays and evaluate functionality of the patterned antibody microarrays, two experimental conditions were designed and immunoassays were performed. In the first experiment, each probe contained one functional antibody with five lanes across a single channel’s width. The same number of lanes was produced in the second experiment while probes in two of the lanes were patterned with two different functional antibodies.
The aim of the first experiment was to demonstrate the capability of the developed technique to produce multiplex protein microarrays with single probe presence at each spot, and the second experiment was designed to show the possibility of producing probes with more than one functional biomolecule at each spot which has recently attracted attention in cell research studies.\(^{32,33}\)

A PDMS stamp with pillars of 25 \(\mu\)m in diameter was used in both experiments to print the APTES solution onto the glass substrate. After forming the microfluidic devices, the cross section of the main channel contained five circular shaped APTES-printed regions. Simulation of the velocity field and concentration distribution was performed prior to the experiments and the results were used to find the optimum experimental conditions for desired patterns. The goal of the first experiment was to pattern protein microarrays in which each probe contains one capture biomolecule (primary antibody). For this purpose, five laminar flow streams containing mouse anti-CD34, rabbit anti-CD31 and rat anti-CD36 primary antibodies were passed through the main channel for 1 h at a flow rate of 2 mm/s. Concentrations of all primary antibodies were 10 \(\mu\)g/mL in PBS.

The second experiment was designed to pattern lanes of five separate functional spots, along the channel width, three of which possessing one antibody of different types and two others containing two individual concurrent antibodies. For this purpose, the primary antibodies were flowed into the main channel at a flow rate of 1 mm/s and the concentration of the CD 36 antibody in the central flow was increased to 30 \(\mu\)g/mL.

After patterning primary antibodies, the channel surface was washed by flowing PBS (10 cm/s \(\approx 50 \text{ dyn/cm}^2\)). Following surface treatment with BSA, to avoid nonspecific binding, FITC conjugated IgG secondary antibody to mouse, Cy3 conjugated IgG secondary antibody to rabbit and Cy5 conjugated IgG secondary antibody to rat, with a concentration of 5 \(\mu\)g/mL each, were mixed and flowed into the patterned channel. In each experiment, about 200 nl of mixed secondary antibodies were incubated on the surface for 30 min in stop-flow to perform a multiplex immunoassay. The surface was then washed with PBS applying the same flow rate used to wash patterned primary antibodies.

2.7. Characterization of Antibody Modified Surfaces. Glass discs of 1 cm in diameter were functionalized with antibodies using a flat PDMS stamp according to the procedure described in section 2.5. X-ray Photoelectron Spectroscopy (XPS) studies were performed with a VG ESCALAB 3 MKII (VG, Thermo Electron Corporation, U.K.) on substrates at various stages of functionalization to identify and quantify the elements on the surface at each stage. Samples were irradiated using an MgK\(\alpha\) source at a takeoff angle of 0° (i.e., perpendicular); analyzed surface was 2 mm \(\times\) 3 mm and the depth sampled was \(\approx 50–100 \text{ Å}\).

2.8. Fluorescence Microscopy. An inverted fluorescence microscope (Nikon TE 2000-E) was used to monitor surface functionalization of the microfluidic platform. Antibodies conjugated with three different fluorescent dyes (Cy3, Cy5 and FITC) were used in the experiments and observed through appropriate filters. All images were captured using a CCD camera (Photometrics CoolSNAP HQ2) and analyzed by MBF_ImageJ (MacBiophotonics, McMaster University).

3. RESULTS AND DISCUSSION

3.1. Characterization of Functionalized Surfaces and Chemical Patterning of Antibodies. Prior to surface patterning of antibodies, the surface of cleaned glass (control), glass microcontact printed with APTES, and APTES printed glass incubated with antibodies were analyzed by XPS (Figure S2, Supporting Information). XPS analysis on APTES printed surfaces showed a marked increase in the nitrogen and carbon peaks compared to the control. Increase in carbon and nitrogen percentage was also observed for antibody functionalized surfaces compared to APTES printed glass. The amount of nitrogen and carbon significantly increased at each step of surface functionalization, which was a clear indication of successful surface patterning using the developed protocol for microcontact printing (Figures S2C and S2D, Supporting Information). The water contact angle of the glass samples was measured before and after cleaning and it was found to decrease from 66 \(\pm\) 0.55° to 3.82 \(\pm\) 0.06° after cleaning. Following APTES printing, the water contact angle for coated surfaces was found to be 40.2 \(\pm\) 0.14°.

Figure 2A depicts patterning of a glass surface with FITC conjugated IgG antibody. To obtain these patterns, a PDMS stamp with pillar shape features (10 \(\mu\)m in diameter with 70 \(\mu\)m spacing) was used to microcontact print APTES in stop-flow conditions. Figure 2B shows Cy3 conjugated IgG antibody patterns in which separate patterns of either 20 \(\times\) 60 \(\mu\)m or 20 \(\times\) 20 \(\mu\)m features with 10 \(\mu\)m spacing were created on the surface using another PDMS stamp. The presence of fluorescence spots on both surfaces indicated that microcontact printing of APTES was successfully implemented to covalently pattern antibodies onto surfaces according to desired micro-meter size features.

XPS results, together with fluorescence labeling, confirmed that the developed experimental protocol for printing APTES on glass substrates was effective. Although microcontact printing of other silanes has already been reported on gold or silicon oxide substrates,\(^{34,35}\) the introduced protocol to microcontact print APTES on glass substrates is rapid and eliminates the requirement for the stringent controls used in conventional APTES coatings such as air-free reaction chambers. In theory, the proposed technique could be further expanded to chemically attach other biomolecules such as proteins, peptides or aptamers.

3.2. Multiplex Antibody Functionalization. Figure 3A represents the microfluidic device functionalized with antibodies through microcontact printing of APTES and microfluidic patterning of biomolecules. Figure 3B shows a cross section of one of the inlet channels, at the boundary of APTES printed...
area with unprinted glass surface functionalized with Cy3 conjugated antibody at a flow rate of 3 mm/s. As displayed in this figure, after washing the surface with PBS at high flow rates, no nonspecific physical adsorption was observed. This is also indicative of strong chemical bonds between the biomolecules and the amine groups in the printed areas.

Figure 3C, D and E shows the fluorescence microscopy images from the surface of the main channel functionalized with FITC, Cy5 and Cy3 conjugated antibodies, respectively. The superimposed image of the three aforementioned images is shown in figure 3F, demonstrating the successful functionalization of the surface with multiple antibodies. Figure 3G depicts the normalized fluorescence intensities of the functionalized surface. The microfluidic design consists of multiple inlet branches, which merge to form the main microchannel (Figure 1E and Figure 3A). These branches join the main channel in different parts along its length, which results in different areas functionalized with three, five and seven antibodies.

These findings address one of the main concerns in using microfluidic patterning, which is controlling the diffusion between laminar flows to functionalize the surface at each area with the desired biomolecules. Although microcontact printing is a useful tool to selectively activate predetermined areas, manipulation of the laminar flows and the concentration of biomolecules in each stream are also determinant factors important for creating the desired arrays of biomarkers. The velocity of the laminar flow streams affects the amount of diffusion of each antibody to the surrounding streams,36–39 and as a result to the microchannel surface (Figure S3, Supporting Information). Diffusion is more important when flat PDMS stamps are used for APTES printing in which the antibodies covalently attach to the whole area inside the channel. This is important in producing region specific patterns through microfluidic patterning.

As depicted in Figure 3G, applying a flow rate of 0.5 mm/s resulted in multiplex functionalization of the surface with considerable diffusion, resulting in more than one antibody presence in some spots. Therefore, for experiments requiring individual probes in specific areas, increasing the flow rate could result in lower diffusion and distinguished boundaries between patterned areas. This is illustrated in section 3.3 involving microarray production. As demonstrated above, surface functionalization could be successfully performed at flow rates up to 3 mm/s. This is one of the main advantages of the proposed method in which diffusion can be controlled by varying flow velocity without altering covalent attachment of the biomolecules.

Interestingly, the diffusion of biomolecules among fluid streams is not the only parameter that can be controlled through flow rate manipulation. The proposed design could very well provide a stable and robust surface functionalization in which the resulting protein patterns possessed high shear resistance. Stability was demonstrated by washing the surface with PBS under flow rates up to 10 cm/s, corresponding to a shear stress of about 50 dyn/cm². Similar devices reported in the literature operated with no more than 1 dyn/cm².4 These results also show that even if region specific patterns or multiplexing are not needed, contact printing of APTES onto glass substrates using flat PDMS stamps can be a promising, rapid and simpler alternative to the conventional silanization techniques in microfluidic devices.

3.3. Immunoassay in a Single Channel Using Multiplex Functional Protein Microarrays. After surface patterning with APTES, multiplex microarrays of primary antibodies were produced and a multiplex immunoassay was
performed. Two sets of experiments were designed and performed as outlined in section 2.6.2. Figure 4B and F depicts the simulation results for concentration distribution among flow streams for each experiment. The areas highlighted with continuous solid lines correspond to the areas patterned with APTES and thus the antibodies in the flow are expected to attach to the surface at these spots. (C and G) Superimposed fluorescence microscope images of the surfaces showing the multiplex immunoassay results. The white dashed lines represent the microchannel walls. (D and H) Fluorescence intensities obtained from C and G in which fluorescently conjugated secondary antibodies are detected by their specific primary antibodies on the surface.

Primary antibodies were patterned on the channel surface as shown in Figure 4A and E. Figure 4C and G shows fluorescence microscopy images of the surface after performing the multiplex immunoassay using secondary antibodies specific to the patterned primary antibodies. In the second experiment, the higher concentration of CD36 (30 μg/mL versus 10 μg/mL) in the central flow stream ensures adequate diffusion of this antibody to the neighboring streams, thus creating columns of probes with dual antibodies in two of the arrays as quantified by fluorescent intensity measurement (Figure 4H).

The multiplex immunoassay results show that the patterned antibodies were functional and that the developed interface can be employed as a multiplex detection interface for high throughput applications using very small sample volumes. Using three different fluorescent dyes conjugated to secondary antibodies, it was possible to show the ability of patterning and detecting five different antibodies across a single channel width with single or dual probe presence at each patterned spot. Such a multiplexed biomarker microarray, with each lane containing a single probe, would be advantageous for high throughput biosensing and point of care diagnosis applications. In addition, the surface patterned with more than one biomolecule in some spots, as illustrated in the second experiment, could be beneficial for some biological applications such as studying cell response to multiple stimuli. The developed microfluidic chip can also be used in applications where high shear stress is required such as adhesion based sorting of lymphocytes on the developed detection interface and investigating their behavior under high shear forces.

4. CONCLUSIONS

A versatile technique for multiplex protein patterning in a microfluidic platform was introduced. This approach consisted in silanization using microcontact printing of APTES onto glass surface followed by microfluidic patterning of multiple antibodies. This led to covalent chemical attachment of different proteinsonto a single microchannel surface, resistant under shear stresses of up to 50 dyn/cm², also allowing operation and manipulation of the microfluidic device at high
flow rates. Since the created patterns were across a single microchannel, confining various analytes to a small surface area in the solution, a multiplex whole immunooassay could be performed using very small sample volumes. This also reduced the number of sensing signals required to be captured for analysis compared to multiple channel devices. Although the proof of concept is shown for antibodies, the versatility and robustness of the approach allows for creating desired patterns of functional biomolecules such as proteins, peptides and aptamers for various applications in multiplex biosensing and whole bioassay systems as well as for cell sorting or cell response to multiple stimuli by simply changing the PDMS stamp design, flow rates and concentrations of biomolecules.

**ASSOCIATED CONTENT**

* Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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