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Title: Microfluidic chip for combinatorial mixing and screening of assays

Actuate-to-Open valves enabled the creation of a microfluidic array chip comprised of picoliter-sized wells with integrated photonic crystal biosensors for combinatorial mixing and subsequent on-chip screening for biomolecular binding events.

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Microfluidic chip for combinatorial mixing and screening of assays

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This paper reports the design, fabrication and validation of a microfluidic well plate for combinatorial screening applications. Each well within the array is comprised of two 200 picoliter compartments that each contain a photonic crystal biosensor to enable the on-chip, *in situ* detection of (bio-) molecular binding events. This microfluidic chip utilizes arrays of Actuate-to-Open valves to isolate all compartments, which allows the chip to be decoupled from pneumatic control lines and thus to be transported freely between filling, sensing and characterization platforms. A proof-of-principle 4 × 4 protein/antibody binding assay was performed to demonstrate the discrete mixing and on-chip sensing capabilities.

Introduction

Microfluidic approaches have been utilized for a wide range of applications including analysis, diagnostics, synthesis, and drug discovery.[1–4] Previous efforts for combinatorial mixing and analysis have focused on continuously flowing microfluidic systems.[5–7] Mixing is performed either by diffusion between contacting streams or by merging droplets in segmented flow, while analysis is performed externally by GC/MS or MALDI-MS. Due to the serial nature of the analyses in these continuously flowing systems, volumes are limited to the nanoliter range, and the complexity of the reaction schemes is limited. To decrease any further in volume while achieving increased reaction complexity, a finely controlled microfluidic approach that allows for on-chip analysis is required.

In prior work we have demonstrated the use of photonic crystal (PC) biosensors as a highly sensitive label-free detection method for performing a wide variety of biochemical and cell-based assays.[8,9] The sensor surface is designed to reflect only a narrow band of wavelengths with close to 100% efficiency when illuminated with white light at normal incidence. A shift of the reflected peak wavelength value (PWV) to longer wavelengths indicates adsorption (*e.g.* binding) of a molecular species on the sensor surface. Recently we have integrated these label-free PC biosensors into polymeric continuous flow microfluidic systems.[10–12] For example, integration of flow channels that originate at individual wells and converge on a row of PC biosensors within a standard 96-well microplate format enabled continuous sampling of each well.[12] This approach was used to determine the binding kinetics of biomolecular interactions. In other work, we also have demonstrated the ability to detect small molecule binding with PC biosensors.[13] This PC biosensor-based technology could be greatly enhanced if combined with a mechanism for synthesis and screening of combinatorial arrays of small molecules, a highly desired capability for a wide range of screening applications. A novel platform with these capabilities would require further reduction of reagent volumes and active, parallel fluid handling to create the combinatorial arrays, while retaining on-chip analysis capabilities.

The advent of microfluidic networks with vast arrays of valves by Quake and coworkers[14,15] has enabled massively parallel chemical syntheses and biological studies in very small volumes (nano- to pico-liters), *e.g.*, protein crystallization screening tools[16] and microfluidic gene expression profiling.[17] These chips are typically fabricated *via* multi-layer soft lithography, the assembly of multiple polydimethylsiloxane (PDMS) membranes that have microscale channels embossed in their surface as negative relief features. Microchannels in the so-called fluid layer are pneumatically filled with liquid using pressurized external feed lines, while microchannels in the so-called control layer are pneumatically actuated at a higher pressure to actively close off fluid lines that they cross at certain locations (Actuate-to-Close or AtC valves). While elegant in routing liquid quanta in microfluidic networks, some aspects of this approach are less desirable with respect to integration of sensing capabilities and the creation of combinatorial arrays. AtC-valve based chips require careful pressure balancing between fluidic and control lines, and with the AtC valves requiring continuous actuation to stay closed, the fluidic layer must be permanently sealed to a substrate to avoid leaking. Furthermore, the chip continuously must be connected to an external pressure source, which limits portability of the chip, *e.g.* between a filling station and detection ancillaries.

As an alternative to AtC valves, Mathies has reported the integration of valves that require vacuum actuation of a flat commercially available PDMS layer sealed between two glass slides with channels etched into both layers.[18] These valves can be utilized in multiplexed arrays[19] and have the distinct benefit of being closed at rest. Like the AtC valves above, irreversible sealing is required for operation. Further increasing the number density of these valves, as required for massively parallel combinatorial applications, would be challenging.

In contrast to the above approaches we report the implementation of massive arrays of vacuum-actuated Actuate-to-Open

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(AtO) valves in elastomeric microfluidic networks, which greatly simplifies chip operation and handling. To highlight the benefits of this approach we created a microfluidic chip capable of metering and mixing a combinatorial array of 200 pL volumes of A × B reactants resulting in A_1B_1 to A_NB_N combinations that are isolated in individual wells. The bottom surface of each well is equipped with a PC biosensor enabling *in situ* detection of biomolecular interactions in each well.

Results

Actuate-to-open valve arrays

In our chips a control layer and fluid layer are utilized similar to microfluidic networks with AtC valves,[14] but fluid lines and compartments are isolated because the AtO valves are passively closed at rest (Fig. 1). The actuation chamber of an AtO valve in the control layer is located directly over a small barrier in the fluid layer that separates adjacent compartments or channels. Upon actuation of the control layer compartment with a negative pressure (on the order of 10 psig below ambient), these barriers lift to open pathways for fluid routing within the chip. Liquid can be pumped into the chip by applying a negative pressure at an opposing outlet. In addition, liquid can be pulled into a dead-ended channel because actuation of the AtO valves gradually removes trapped air in the fluid channel through the gas-permeable PDMS membrane into the control layer. After the negative pressure is released, the barriers between compartments collapse shut, back into the rest state. The pneumatic lines can then be disconnected from the chip without affecting the sealed, reagent-filled compartments within the chip.

As mentioned above, a pressure of about −10 psig is needed to overcome the adhesion between the valve seat and the glass surface, thereby opening the AtO valves. A much lower pressure, down to −1 psig is sufficient to keep AtO valves open. In the rest state (closed), the AtO valves can easily sustain liquid pressures up to 10 psig.

Use of AtO valves as opposed to AtC valves in elastomeric microfluidic networks has many advantages: (i) AtO valves are closed in rest, the state most valves are in most of the time in just about any application. Compared to the AtC valves, this eliminates the need for continuous actuation at pressures of 5 to 30 psig; (ii) They eliminate the need for irreversible sealing of the fluid layer to a bottom substrate such as a sensor surface (Fig. 2(a)); (iii) The gas permeable properties of PDMS eliminate the need for reagent feed lines as fluids are pipetted over inlet
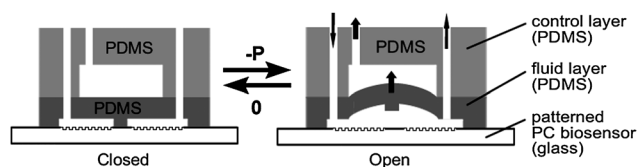
Fig. 1 Schematic of an Actuate-to-Open (AtO) valve comprised of a pneumatic control layer and a fluid layer, placed on a PC biosensor patterned on a glass substrate. Actuation by applying a negative pressure (right) lifts the barrier between adjacent compartments, allowing for fluid flow. After release of the negative pressure the valve collapses to the closed rest state (left).
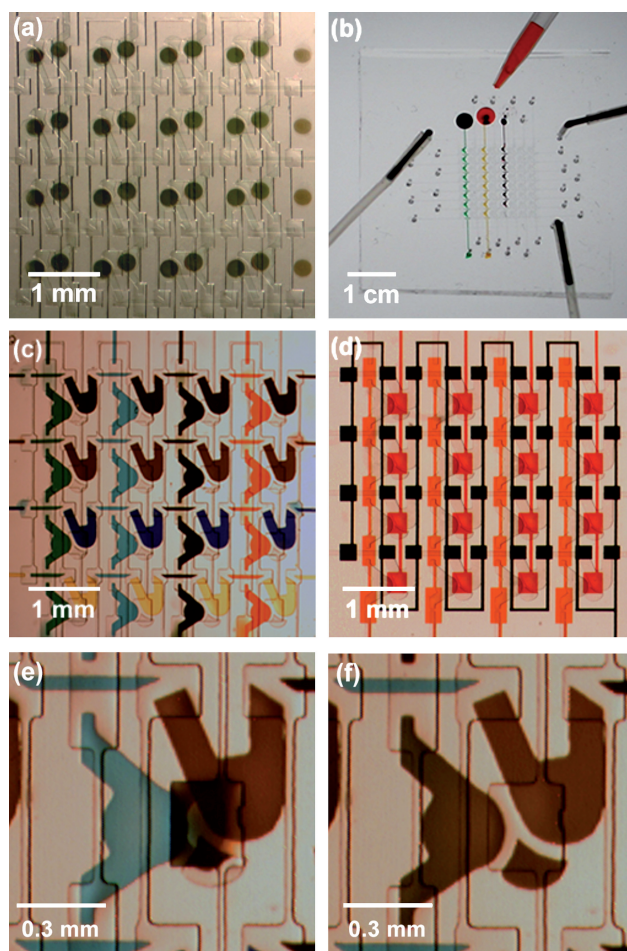
Fig. 2 (a) Optical micrograph of a 4 × 4 microfluidic chip placed on a patterned array of biosensors. Each well is comprised of two adjacent compartments, each with their own biosensor (dark circles). (b) Photograph of the multilayer microfluidic array chip. Dyed droplets are pipetted at the inlet ports and sucked into the columns by actuation of the appropriate set of valves. (c) Optical micrograph of a 4 × 4 array of wells, highlighting the fluidic layer: the 4 rows and 4 columns are each filled with an aqueous solution of different color to show the combinatorial generation of 16 different combinations of reagents. (d) Optical micrograph of a 4 × 4 array of wells, highlighting the control layer: The 3 sets of valves control filling of the horizontal rows (black squares), filling of the vertical columns (orange rectangles), and the mixing of reagents in adjacent compartments (red squares). (e) Close up of an individual well in the chip at the very onset of mixing (valve actuated). (f) Mixing is completed following repeated valve actuation over 3–5 minutes.

ports and are pulled into the fluid lines upon actuation of AtO valves, dramatically reducing dead volume (Fig. 2(b)); (iv) In stark contrast to AtC valve-based chips, an AtO chip is highly portable since it can be disconnected from all lines after filling, and moved to a detection platform (*e.g.* microscope, plate reader); and (v) The AtO valve design affords a higher degree of complexity, *i.e.* a higher density of compartments per unit area because valve area overlaps with compartment area as opposed to being located between compartments.

Constant pneumatic actuation is not required for AtO-based microfluidic systems, making them more amenable for field-portable applications. The absolute pressures needed to actuate

AtC or AtO valves are similar and can be achieved in a portable device, for example, using gas tight syringes. However, AtO valves only need to be actuated briefly during filling of a chip (typically less than 30 seconds), whereas AtC valves need to be actuated over the course of an experiment (*e.g.*, an incubation over several minutes to hours) in order to maintain the liquids confined in separate compartments. The gas permeable nature of the PDMS leads to a gradual loss of pressure on AtC valves which a comparably portable system would need to account for.

Design and fabrication of the PC biosensor array chip

We created a 4 × 4 microfluidic well plate with combinatorial mixing and sensing capabilities as enabled by the use of AtO valves (Fig. 2(a) and (b)). Each well is comprised of two U-shaped, 200 pL compartments (Fig. 2(c) and (e)). Three separate sets of AtO valves control bi-directional filling and mixing of all 16 wells. The reagents (2 µL each) are pipetted at the inlets of each of the four rows and four columns. Then actuation of the first set of valves (Fig. 2(d), black squares) initiates horizontal flow; filling the rows comprised of the right compartment of each well. Subsequent actuation of the second set of valves (Fig. 2(d), orange rectangles) initiates vertical flow; filling the columns comprised of the left compartment of each well. Upon releasing pneumatic actuation, all valves collapse into their rest state, thereby isolating the reagents in their individual U-shaped compartments. Next, by actuation of the third set of valves (Fig. 2(d), red squares), reagents in all 16 sets of two adjacent U-shaped compartments are mixed. Mixing occurs by diffusion and convection upon 8–15 valve actuations and relaxations over the course of three to five minutes, as demonstrated with dyes (Fig. 2(e) and (f)).

Next we created a patterned 4 × 4 array of two circular PC biosensors (D = 250 µm) on glass (Fig. 2(a)). The porous, dielectric-based PC biosensor gratings were obtained *via* replica molding as reported previously,[11] and photolithography was used to create the patterned array of circles. This pattern exactly maps on the dimensions and relative spacing of the U-shaped compartments of the fluid layer and avoids intra-well leaking, which occurred initially along the grating ridges when using unpatterned, uniform PC biosensor substrates.

Imaging configuration

The PC biosensor imaging capability is based on detecting changes in reflected PWV as a function of spatial position, where regions with the greatest density of biomolecular interaction will result in the greatest wavelength shift.[8] White light illuminates the PC biosensor surface at normal incidence with the polarization perpendicular to the sensor grating lines. The reflected light is directed through a beam splitter and an imaging lens to a narrow slit aperture at the input of the imaging spectrometer. Using this method, reflected light is collected from a 9.1 mm line on the sensor surface, while the width of the imaged line is determined by the width of the entrance slit of the spectrometer. The imaging spectrometer contains a 2-D CCD chip (Acton Research) in which the line image is divided into 512 pixels. A spectrum with a resolution of 2048 wavelength data points is acquired for each of the 512 pixels imaged. Upon peak-finding

analysis of all 512 spectra, the PWVs for each of the 512 pixels are determined, and thus a line of 512 pixels is generated for the PWV image of the PC biosensor. The PC biosensor is translated with a motorized stage in a direction perpendicular to the image line in small increments to generate a 2-D spatial PWV image of the PC biosensor. The spatial separation of the image lines is determined by the step size of the stage between each image line acquisition (here 22.3 µm).

Binding assay

To demonstrate the combinatorial capabilities of the PC biosensor array chip, we performed a proof-of-principle protein/antibody binding assay. The high sensitivity of the PC biosensors requires a method that effectively distinguishes nonspecific binding of antibodies to a sensor surface from an antibody adhering to a specific, surface-immobilized protein. To that end, we incorporated an experimental and a control compartment for each well within the array, with each compartment having its own PC biosensor. Reagents are introduced into the microfluidic network by actuation with a negative pressure, and when the AtO valves are relaxed, all compartments are sealed off, so the chip can be transported for further analysis.

For the binding assay experiment, proteins A and A/G (Pierce Biotechnology) at 0.5 mg/mL in Phosphate Buffer Saline (PBS, Sigma-Aldrich), were incubated for 10 minutes in the experimental compartments (2nd, 4th row of Fig. 2(c)). Sea Block (Pierce Biotechnology) diluted in PBS to 20% by volume was then incubated for 10 minutes across all compartments as a preventative measure against nonspecific binding. Subsequently, all compartments were rinsed with PBS, and goat, chicken and human immunoglobulin G (IgG) antibodies (Sigma-Aldrich) at a concentration of 0.5 mg/mL in PBS were incubated for 10 minutes in the control compartment of each well within the 2nd, 3rd and 4th columns. PBS was introduced in the 1st column as an additional control. After introducing antibodies, the valves were disconnected from the pneumatic lines and relaxed to the closed rest state, sealing off all filled compartments.

At this point, each well contained either PBS or an antibody solution in the left (control) compartment, and PBS in the right (experiment) compartment. The chip was disconnected from all pressure lines and a background scan was taken to record the index of refraction of PC biosensors covered with certain proteins and in the presence (or absence) of certain antibodies in PBS. Next, the chip was reattached to the negative pressure source, and the contents of adjacent control and experiment compartments was mixed by actuation of the mixing valves as explained above (Fig. 2(e) and (f)). A 30 minute incubation period with the mixing valve open followed, allowing the antibodies to equilibrate with the surface-immobilized proteins, particularly those in the experiment chamber. The chip was disconnected from the vacuum source and a final scan of the whole array was taken. Subtraction of the background yielded the binding assay results shown in Fig. 3. Red color indicates strong binding between a given protein and antibody combination. The lack of red coloring in the internal controls, as well as the microfluidic rows and columns containing only PBS indicates lack of nonspecific binding as well as a lack of
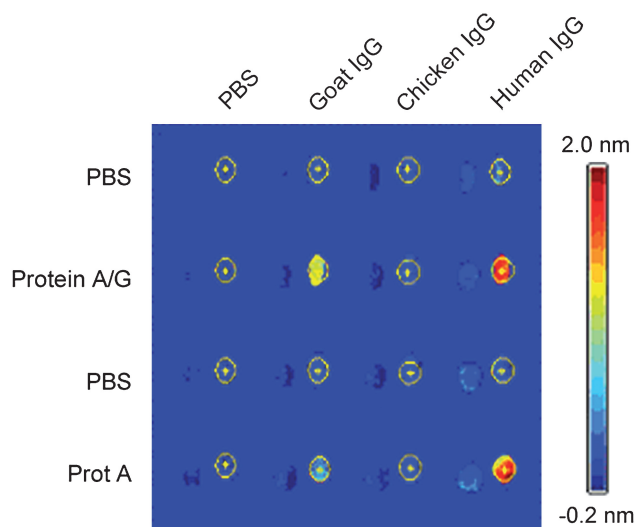
Fig. 3 On-chip binding assay between proteins (A and A/G) and antibodies (goat, chicken and human). The binding experiment between each protein–antibody combination takes place in the circled compartment on the right of each well which is compared against a control compartment to its left to rule out nonspecific binding of antibodies. Red coloring within these circles indicates a shift in the PWV, indicative of a binding event.

intra-compartmental leaking, which should have been immediately noticed with the protein–antibody assay with such high binding affinities used here. While the polymeric device is only reversibly sealed to the sensor surface, the AtO valves in rest were sufficient to prevent leaking.

Finally, the data was analyzed to quantify the extent of binding for each protein–antibody combination. The pixels within each experiment compartment (indicated by the circles in Fig. 3) were averaged to a single PWV shift value. An average PWV shift for each protein–antibody combination was obtained by subtracting the average PWV of a control compartment from the average PWV of each adjacent experiment compartment to account for signal drift across the array (Fig. 4). The measured PWV shift values we obtained here are consistent with protein/IgG binding experiments previously published[12] where Human
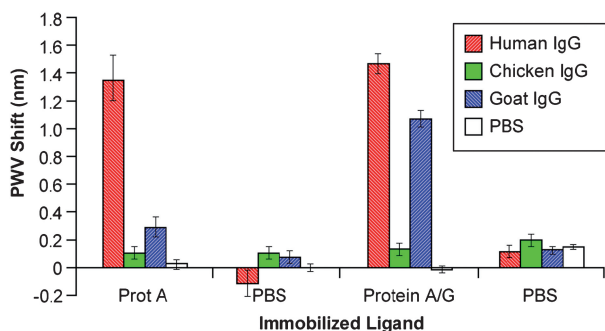
Fig. 4 Analysis of PWV shifts observed in on-chip binding assay. As expected, Human IgG binds strongly to protein A and protein A/G, while Goat IgG has a weaker affinity for these proteins. Chicken IgG exhibits a response similar to PBS (± 0.2 nm), indicative of the lack of binding affinity, as expected.

IgG has a strong binding affinity for both Protein A and A/G, Goat IgG has weak binding affinity for Protein A but strong affinity for Protein A/G, and chicken IgG lacks binding affinity with either protein A or A/G.

Conclusions

Here we showed the design and fabrication of a microfluidic array chip with combinatorial mixing and on-chip sensing capabilities. A combinatorial matrix of reactions can be performed within 200 pL microfluidic compartments that can be sealed reversibly to a patterned PC biosensor for the determination of binding events. The ability to decouple such AtO-valve based chips from an external pressure source while retaining the reagents locked up in isolated compartments, greatly simplified their use, particularly for sensing purposes that need to be performed away from fluidic handling capabilities. Current valving technology cannot accomplish this level of post-mixing portability at such high densities. A common fear when using reversibly sealed microfluidic chips is their propensity to leak, but the proof-of-principle reaction scheme shown here using IgG-antibodies demonstrated that AtO-based microfluidic networks do not suffer from leaking issues. Binding events properly correlated to expected results, showing a high fidelity within wells despite the reversible seal. The ability to perform these reactions reliably at such small scales with such ease of use will serve as a great advantage to performing vast array chemistries using much smaller amounts of reagent in combinatorial matrices than presently used in traditional micro-titer plate approaches. Furthermore, the inherent ability of PC biosensors to detect small molecule binding events[13] is preserved in the microfluidic platform presented here. This microfluidic array chip is thus a promising candidate for chemical synthesis and combinatorial screening applications where multiple steps have to be carried out in parallel in minimal reagent volumes.

Acknowledgements

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