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Rapid, multiplexed microfluidic phage display

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The development of a method for high-throughput, automated proteomic screening could impact areas ranging from fundamental molecular interactions to the discovery of novel disease markers and therapeutic targets. Surface display techniques allow for efficient handling of large molecular libraries in small volumes. In particular, phage display has emerged as a powerful technology for selecting peptides and proteins with enhanced, target-specific binding affinities. Yet, the process becomes cumbersome and time-consuming when multiple targets are involved. Here we demonstrate for the first time a microfluidic chip capable of identifying high affinity phage-displayed peptides for multiple targets in just a single round and without the need for bacterial infection. The chip is shown to be able to yield well-established control consensus sequences while simultaneously identifying new sequences for clinically important targets. Indeed, the confined parameters of the device allow not only for highly controlled assay conditions but also introduce a significant time-reduction to the phage display process. We anticipate that this easily-fabricated, disposable device has the potential to impact areas ranging from fundamental studies of protein, peptide, and molecular interactions, to applications such as fully automated proteomic screening.

Proteomics is an emerging field involving the study of protein functions, activities, and interactions.[1,2] In the last several decades, automated genomics technologies have led to high-throughput, low cost, and rapid DNA sequencing. By contrast, access to proteomic tools has been more restrictive due to their scale and expense.[3] Automated robotic technologies have revolutionized biological research by allowing much faster processing of large analytical assays,[4] although such systems are often limited in portability and require minimum working volumes.[5,6] By contrast, microfluidic systems allow for reduced sample sizes, thereby rendering chemical and biological assays more efficient and portable.[7] An impressive variety of biological technologies has been incorporated into microfluidic chips, including protein crystallization,[8] polymerase chain reaction (PCR),[9] and enzyme-linked immunosorbent assays (ELISA).[10] Such chips have been designed with cell culture,[11] antibody,[12] genetic,[13] and drug delivery[14] studies in mind. However, relatively few automated lab-on-a-chip technologies have been developed for proteomic screening.[15–17]

Phage display is a powerful method for target-specific determination of molecular interactions, *via* the exploitation of discriminative affinity selection. Further, its versatility has allowed studies of peptide binding interactions against substrates as varied as metals,[18] minerals,[19,20] small molecules,[21,22] polymers,[23] and nanomaterials.[24,25] In the phage display protocol, a direct phenotype-genotype linkage provides for combinatorial screening of binding affinities toward specified targets *via* an *in vitro* selection process termed biopanning. For conventional selection processes, a target molecule is immobilized onto a substrate, and subsequently incubated with a library of phage-displayed peptides. Non-binding phage are washed away with buffer while binding phage are collected using an acid elution step. This is then followed by bacterial titer and amplification, and at least two additional rounds of selection. After the final round of biopanning, eluted phage are grown on agar plates, and individual plaques are selected for DNA characterization to determine the amino acid sequence of the phage-displayed peptides. While sufficient for studying a single target, this process becomes time-consuming, labour intensive and cumbersome with multiple target molecules. Although recent studies on biopanning in large-scale fluidic devices have been shown, they do not address the more time intensive components of the process and thus are not any more amenable to high-throughput multiplexing.[26,27] Here we report for the first time a time-efficient microfluidic approach that allows for simultaneous single-round identification of binding sequences for multiple targets, without any need for bacterial culture.

A device made entirely of silicone (polydimethylsiloxane, PDMS) simplifies fabrication, while rendering the completed chip flexible, portable, and disposable. A schematic of the functional components of the device is outlined in Fig. 1. Consisting of three PDMS layers (Fig. 1a), the phage display target is immobilized on the base layer using established silicone surface chemistry modification techniques (Fig. 1b).[28,29] The middle tier contains channels through which reagents flow, and is capped on top with channels which function as valves and pumps controlling fluid flow. When pressurized gas enters the control channels, the thin polymer membrane between the upper two layers deflects downwards to obstruct further flow in the middle layer (Fig. 1c). Once the pressure is lifted, the membrane rapidly

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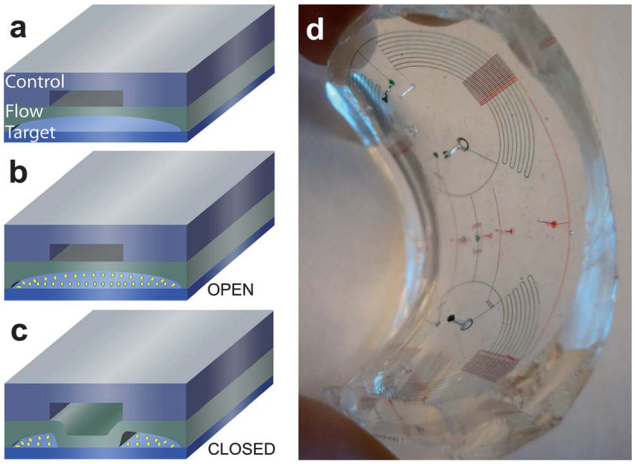


Fig. 1 Elastomeric, multiplexed phage screening device. (a–c) Schematics of (a) the trilayer device, with Control, Flow, and Target layers labelled accordingly, (b) Target channel functionalization, and (c) Valve-driven biopanning. (d) Photograph of completed device, with green and red food dye representing flow and control channels, respectively.

returns to its original position and fluid may commence circulation. Unlike other microvalves, this design allows for rapid response time, minimal leakage, and ease of fabrication.[30] Small actuation forces produce large membrane deflections, and complete sealing of channels is readily attained.

Indeed, these membrane deflections allow for efficient biopanning. When control channels are serially arranged and pressure is cyclically applied and released, a peristaltic pump is formed, wherein the flow rate is controlled by the actuation frequency. Previous studies have shown that similarly designed pumps can accelerate kinetics by at least 60 times.[31,32] Here, the peristaltic pump is used to agitate solutions in the flow channel, mimicking the mixing and shaking motions employed during bulk phage display panning. Finally, the design contains an inlet into which samples and buffers are introduced, an outlet from which desired phage are collected, and a waste reservoir. A photograph of a completed flexible device is shown in Fig. 1d.

Fabrication of the device follows standard lithographic protocols for the flow and control layers. Positive photoresist, SPR 220-7 (Shipley Corp., Philadelphia, PA) was spin-coated to a height of 15 μm for the flow layer before being heated to 120 °C to allow the photoresist to reflow and form the rounded shape necessary for complete channel closure. Negative photoresist, SU-8 2015 (Microchem Corp., Newton, MA) was spin coated to a height of 25 μm for the control layer. Room-temperature vulcanized PDMS (GE Silicones, Waterford, NY) was used to create elastomeric replicas. A 20 : 1 (PDMS:crosslinker) mixture was used for the flow layer and a 5 : 1 mixture was used for the control layer. The flow layer mixture was spin-coated to produce a 30 μm thin elastomeric layer while the control layer mixture was poured to a height of 2 mm. Both layers were baked separately at 80 °C for partial curing before the control layer was removed from its master and perforated to create inlets for the pressure source. This top layer was aligned with the flow layer and the two were cured together for an additional 30 min. The two-layer assembly was then removed from the flow master and perforated to create injection ports in the fluid layer. Lastly, this was brought into contact with a flat slab of PDMS to complete the tri-layer device. The chip was interfaced with a nitrogen gas pressure source *via* solenoid microvalves (Parker Hannifin Corp, Hollis, NH) and Tygon tubing (Cole-Parmer, Vernon Hills, IL), and on-off sequences of valves and pumps were governed by a programmable function generator. Flow channels were 100 μm wide, 10 μm tall, and had an overall volume of about 0.1 μL.

To test device functionality, a control target and three lesser-studied targets were simultaneously screened. Streptavidin was chosen as the robust control, as its consensus sequence – the HPQ motif – has been well-established by existing phage display studies.[33] The other targets chosen were tumor necrosis factor alpha (TNF-α), interleukin-4 (IL-4), and interleukin-5 (IL-5), all of which are important biomarkers in the pathology of asthma,[34,35] and for which consensus sequences have not been firmly established. Channel design was streamlined such that multiple valves and pumps were controlled by a single switch, according to the streamlined CAD design shown in Fig. 2. Reagents common to all four targets, including water, lysine, PBS, BSA, TBST, and the library itself, stemmed from the same inlet, but each target had distinct waste reservoirs and outlets to prevent cross-contamination.

Microscreening proceeded as follows. First, a surface modification was applied to the entire device, according to the reactions in Scheme 1. Specifically, the channels were flushed with water, incubated with 0.01% p-L-lysine, and rinsed with buffer. After a subsequent incubation of 12.5% glutaraldehyde, targets were introduced and allowed to sit for 1 h at room temperature. This was followed by incubation of 40 mM ethanolamine solution and another buffer rinse.[28] Next, the device was coated with 5 mg mL^{-1} BSA to prevent nonspecific binding prior to introducing the phage library. Phages were agitated in the channels for 1 h using the peristaltic pump before nonbinding virions were flushed into their respective waste reservoirs using Tris-buffered saline and 0.5% Tween-20.

Critically, a key departure from the conventional phage display protocol was employed at this point in order to significantly reduce overall time of the device operation. Rather than elute target-binding phage with acid, a lysis buffer (10 mM Tris-HCl, pH 8.0, 100 mM EDTA, 1% SDS) was introduced to release DNA from within the remaining target-bound virions. The DNA-containing solution for each of the separate targets was collected from the microfluidic device and added to a PCR amplification mixture of REDTaq polymerase (Sigma Aldrich, St. Louis, MO), forward primer (5′-CCTCGAAAGCAAGCTGATAAC-3′), and reverse primer (5′-GTACCGTAACACTGAGTTTCG-3′).[36] The mixture was placed in a thermocycler and subjected to 25 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. Each of the four PCR

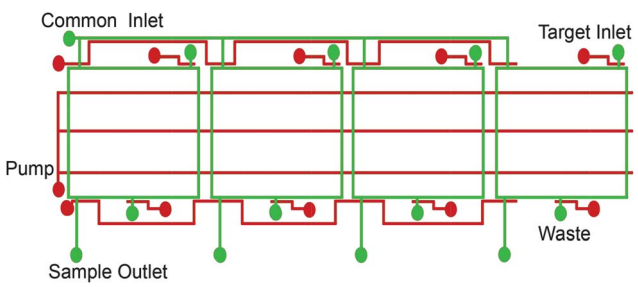
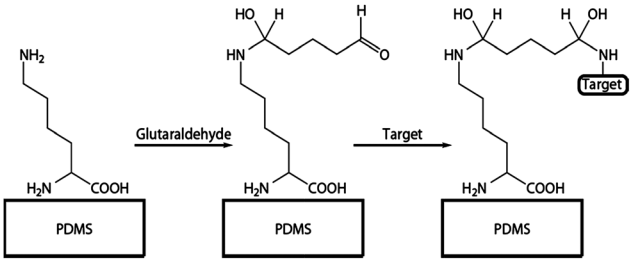


Fig. 2 Simplified design of the multiplexed device, illustrating inlets and outlets. Flow layer is shown in green, control layer in red.



Scheme 1 Surface modification of PDMS for target immobilization.

Table 1 Peptide sequences identified from the single-round, PCR-amplified microfluidic chip

Target	Sequence
Streptavidin	NPWDEFRTHHPQ
TNF-α	NNNKPNPHELHR
IL-4	NNDHARHLNYHS
IL-5	YMGMTKHNIYAQ

products was separated *via* electrophoresis on a 1.2% agarose gel, with a single 0.33kb DNA amplimer confirming presence of phage DNA. Gel extraction was performed and purified DNA for each sample was characterized *via* automated sequencing methods.

Results of these single-round, multiplexed microfluidic panning experiments are shown in Table 1. For streptavidin, the phage-displayed peptide contained the HPQ motif at the C-terminal position, typical for the type of phage library used,[27] thereby validating our experimental approach. This result is significant because it demonstrates that multiple rounds of biopanning and bacterial culture may not be necessary to identify a specific binding peptide in the microfluidic system. In parallel, sequences of the binders for TNF-α, IL-4, and IL-5 were found, as shown in Table 1.

An earlier study had shown that lysing target-bound phage, rather than eluting them, can result in the identification of unique sequences overlooked by conventional biopanning.[36] This is due to the fact that high-affinity phage-displayed peptides are often left behind even after extended acid elution, and can be further diluted by faster amplifying phage during successive rounds of bacterial amplification and selection. Our results extend upon this study by eliminating not only the need for bacterial amplification, but also the need to clone PCR products into a vector and subsequently select individual clones for sequencing. In lieu of these steps, our optimized microfluidic panning approach, followed by PCR amplification allows for the direct detection of a single phage particle. In other words, not only does the microfluidic process obviate bacterial titers and amplification, but it also renders sequencing of individual phage clones unnecessary. These simplifications dramatically reduce the overall time and labour intensity of the phage display protocol, as outlined in Table 2. Overall, this results in a *ca.* four-fold time reduction overall per target, as compared to conventional phage display – a result that becomes more significant with each additional target added. Indeed, for 4 targets, this results in a net 15-fold reduced time investment per target relative to conventional phage display screening.

Table 2 Time of conventional and microfluidic phage display

Step	Conventional	Microfluidic
Microfluidic Fabrication	—	4 h
Target Immobilization	1 h	3 h
Blocking	1 h	1 h
Wash	10 min	30 min
Biopanning	1 h	1 h
Wash	10 min	1 h[27]
Elution	1 h	—
Lysing	—	1 h
Bacterial Amplification & Phage Titer	16 h	—
2nd, 3rd Rounds Biopanning	20 h × 2	—
Purification for Sequencing	3 h	—
PCR Amplification	—	4 h
Total Time	63 h	16 h
Total Time per 4 Targets	**63 h**	**4 h**

Conclusions

We have created a microfluidic chip capable of identifying high affinity phage-displayed peptides for multiple targets in just a single round, without the need for any bacterial culture. The performance of the device was confirmed *via* a control streptavidin target, while simultaneously screening for three additional important biomarkers. Not only does the device result in a significant fifteen-fold time reduction and a ten-fold volume reduction per target, it is also more sensitive – picking up sequences otherwise lost during conventional phage display. Given that phage display is inherently a high throughput assay, we anticipate that such a device would be a powerful tool in molecular binding studies. Further, the disposable nature of the device eliminates any possibility of cross-contamination. The versatility of the target layer chemistry suggests that the device can be similarly used to rapidly screen against other biomarkers,[37] and a host of other materials, including metals,[38] viruses,[39] bacteria,[40] and polymers.[41] Indeed, this development has important implications for many fields, including synthesis of protein nanomaterials, where sequence and arrangement of peptides can be used to optimize the activity of nanotubes,[42] nanoparticles,[43] and other nanostructures.[44] In future studies, we plan on 1) extending the multiplexed design to wafer-scale arrays, allowing for more targets in parallel assays, 2) further automating the process with software and controls, and 3) incorporating the PCR step directly into the device *via* microfluidic PCR modules.[45] While we were able to pinpoint single target-binding peptide sequences in this study, it is possible that other targets may have more than one binding sequence associated with them. Consequently, next generation DNA sequencing can be used to characterize cohorts of phage clones.

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